THE EFFECT ON RAT GROWTH OF ALTERNATED PROTEIN INTAKES

ROBERT A. HARTE, JOHN J. TRAVERS AND PETER SARICH
Research Laboratories, The Arlington Chemical Company, Yonkers 1, New York

ONE FIGURE

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Since the ultimate aim of much animal experimentation is the procurement of data applicable to man, experimental designs formally analogous to those common to human habit are of continuing interest. An initial experiment motivated by this concept has already been reported (Harte, '47).

Most small animal nutrition experiments are carried out with diets of constant composition. Human dietary practice, on the other hand, except, perhaps, in earliest infancy includes varied diet composition. To mirror human dietary vagaries experimentally would be an endless task; to simulate approximations within severely limited spheres is readily attainable.

Day-to-day variations in human food habits are innumerable. Not only may the several components ingested vary in quantity but also in quality. Further, numerous dietary components probably interact; many, no doubt, in ways not yet elucidated. These interactions are, in all likelihood, conditioned variously by the make-up of the diet as a whole.

This report presents data obtained from rat growth studies in which animals were fed, on alternate days, isocaloric rations containing different quantities of the same protein; in 1 experiment, protein quality as well as quantity was alternated.

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EXPERIMENTAL

Groups of randomly chosen weanling male rats maintained under conditions similar to those previously described (Harte et al., '47) were fed, *ad libitum,* rations made up to contain differing quantities of protein. Ration composition with respect to fats, salts, and vitamins was the same as that reported before; approximate caloric equivalence was attained by appropriate adjustments in content of cornstarch.

In 4 series of experiments, weighings were made every sixth day for 30 days; in the fifth series, weekly weighings were made for 4 weeks. The animals were not pre-conditioned by acclimatization to any synthetic ration since this practice has previously been shown to offer, at best, questionable advantage (Harte et al., '47).

Food spillage was accounted for by scrupulous daily collection. The amount so collected was deducted from the apparent consumption of each ration and the protein intakes were calculated from the net food intakes.

RESULTS

Examination of the data summarized in the upper part of table 1 shows that growth responses were virtually identical in the various comparisons involving different levels of the same protein. In the table, the first line of each pair of entries is to be taken as the control for the second. The 2 levels indicated in the second line of each pair represent the protein levels in the rations which were alternated; the control level, in every case, is the mean of the alternated levels. The growth curves in these instances coincided well throughout the experimental period and are not, therefore, reproduced.

On the other hand, the data in the lower part of table 1, where the animals in the alternated feeding group received not only different quantities of protein in the ration but protein differing in quality as well on alternate days, showed markedly different responses. The growth curves obtained under these circumstances are shown in figure 1.

TABLE 1

Growth responses of rats offered alternated diets.

	RATION FED	NO. OF ANIMALS	INITIAL WEIGHT	WEIGHT GAIN	FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY [1]
			gm	*gm*	*gm*	*gm*	
			Rations containing constant quality protein at different levels				
Casein [3]	7% (control)	7	53.6 ± 2.0 [2]	32.6 ± 3.3	227 ± 12	15.9 ± 0.8	2.02 ± 0.15
	5% and 9%	9	54.9 ± 1.9	32.8 ± 5.1	229 ± 11	16.2 ± 0.8	1.96 ± 0.21
	10% [4] (control)	8	64.5 ± 2.4	61.6 ± 5.9	272 ± 19	27.2 ± 1.9	2.24 ± 0.12
	5% and 15% [4]	8	57.4 ± 3.6	60.9 ± 2.1	268 ± 9	27.7 ± 0.8	2.20 ± 0.08
	18% (control)	10	54.0 ± 2.1	108.4 ± 6.9	325 ± 12	58.5 ± 2.2	1.85 ± 0.08
	12% and 24%	10	54.4 ± 1.6	115.1 ± 6.0	319 ± 13	57.2 ± 2.3	2.02 ± 0.07
Wheat gluten	20% (control)	10	53.8 ± 1.2	9.0 ± 2.2	175 ± 11	35.4 ± 2.2	0.239 ± 0.06
	10% and 30%	10	51.4 ± 1.2	4.3 ± 1.9	162 ± 9	31.3 ± 1.8	0.121 ± 0.06
			Rations containing protein mixtures of varying quality at different levels				
Wheat gluten, 10% + hydrolysate,[5] 5%		9	48.3 ± 2.2	65.4 ± 6.0	298 ± 19	44.7 ± 2.8	1.45 ± 0.07
Wheat gluten, 10% alternated with wheat gluten, 10% + hydrolysate, 10%		10	53.9 ± 1.9	46.7 ± 5.3	236 ± 13	35.4 ± 1.9	1.29 ± 0.12

[1] Protein efficiency is the ratio of weight gain to protein consumed.
[2] Indicated limits represent the standard errors of the means.
[3] Labco, from Borden Co.
[4] These tests were of 28 days' duration; the others continued for 30 days.
[5] The hydrolysate used was aminoids, a protein hydrolysate product manufactured by The Arlington Chemical Company.

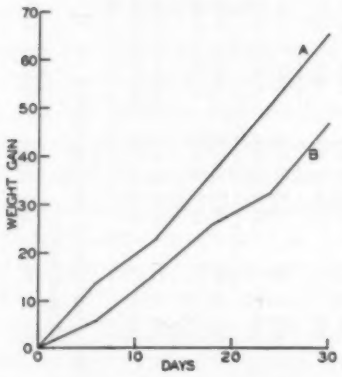

Fig. 1 Weight gain vs. time curves for animals on basic 10% wheat gluten diets supplemented (A) daily with 5% hydrolysate and (B) on alternate days with 10% hydrolysate.

DISCUSSION

The curve relating the variables protein efficiency and per cent casein in the ration is known to have a maximum at about 10–12% casein (Osborne, Mendel and Ferry, '19, and others). In view of this it is, perhaps, not surprising that alternations centered about a low level (7%), or a high level (18%) are without effect, as measured by weight gain or protein efficiency, when compared with appropriate controls. In the former case all of the levels are on the ascending portion of the curve; in the latter, on the descending side. On the other hand, it is hard to find an *a priori* explanation of the results of alternation about the 10% level, where the alternated levels are on either side of the maximum.

These data suggest that when a reasonably complete protein is fed, the rat has the capacity of integrating its intake over a 48-hour period. This finding is in marked contrast to that reported by Cannon et al. ('47) who found that a rat could not integrate its food supply when a missing amino acid was supplied 1 hour later, and to that of Elman ('39) who found that the dog could not utilize an intravenously administered

acid hydrolysate of casein when the missing tryptophane was introduced after 6 hours.

Another explanation which might be considered rests on the observation of Allison and associates ('46) that the nitrogen balance index of proteins is higher in depleted dogs than in normal animals. It is likewise true that depleted rats utilize protein more efficiently for growth (Travers and Harte, '47). The animals that were offered 5 and 15% casein might be regarded as being alternately depleted and repleted. The observed protein efficiency of this group is higher than might have been predicted on the basis of the protein efficiency vs. protein level curve for casein. If the protein intake on the 5% ration is inadequate to sustain the accelerated growth rate of the alternated 15% ration, then there would result an intermittent pseudo-depletion with an intermittent, concomitant better-than-normal utilization of the protein fed at the higher level. Frazier et al. ('47) have found that, in general, the effects of an incomplete amino acid ration are realized within 24 hours. So, also, a low level of protein, contrasted with an alternate high level, could be expected to manifest its effects in a similar period of time, but the overall effect, in this case, seems to be integrated over the unit interval of 48 hours.

That no significant effect was found in the wheat gluten experiment might have been anticipated from the report of Barnes et al. ('45). Our experiments are uncomplicated to the extent that the nature of the protein source was not varied in the several comparisons, merely the level. Where daily variation introduces the factor of differing quality as well, the possibility of other results could be entertained. This variation in quality as well as quantity constitutes a closer analogy to human habits.

The experiment summarized in the lower part of table 1 and figure 1 compares the response of the growing rat to a constantly supplemented poor protein diet (10% wheat gluten) with the response to intermittent supplementation of the same diet. To some extent this might simulate conditions apt

to prevail in the actual use of dietary protein supplements in medical practice. Here the efficiency of utilization for growth of dietary protein ($N \times 6.25$) was not affected; the protein efficiencies do not differ significantly. However, the actual growth responses do show substantial divergence which is statistically significant ($p = 0.03$).

For the protein hydrolysate product employed, an amino acid analysis (Block, '47) shows that its limiting amino acids are methionine and cystine (Block and Mitchell, '46–'47), while pair-fed weanling rats do as well on this material as on casein (Oser, '47). The deficiency of lysine in wheat gluten is well-known. In the alternation experiment then, it may be that the situation is more nearly akin to that observed by Cannon and coworkers ('47) and we are encountering a (partial) manifestation of the rat's inability to integrate a complete amino acid supply when all of the necessary amino acids are not offered simultaneously. This problem has recently been explored in the case of growing rats by Geiger ('47). He found that delayed supplementation of an incomplete protein by the missing amino acid (12-hour alternation) failed to promote growth.

It is noteworthy that the animals in the alternated group shown in the lower part of table 1 consumed hydrolysate almost exactly equivalent to one-third of their total protein intake, just as did the rats in the control group. The difference in total food intake in these groups is statistically significant ($p = 0.02$) and this may well be the effect of the alternation and thus the cause of the difference in growth response.

SUMMARY

1. Weanling rats grow as well, and utilize dietary protein with the same efficiency for growth whether it is supplied at a constant level in the diet or whether the level is alternated daily about the same value as a mean, provided the protein quality is constant (casein or wheat gluten).

2. Daily alternation of both quality and quantity of dietary protein, using wheat gluten and a protein hydrolysate, results

in significantly poorer growth response but not significantly lowered protein efficiency.

3. The data suggest that prescribed dietary protein supplementation should be maintained constant to achieve maximum effectiveness from a given amount of supplement.

ACKNOWLEDGMENT

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THE DIGESTIBILITY OF RAPE-SEED OIL IN THE RAT[1]

HARRY J. DEUEL, JR., AMBER L. S. CHENG AND MARGARET G. MOREHOUSE

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

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There is general agreement that vegetable and animal fats which melt at 50°C. or lower are practically completely digested in man and in other animals. The chief exception to the complete digestibility of liquid fats in man is in the case of such irritants as castor or croton oil where the premature removal of the fat from the small intestine before the completion of lipolysis and absorption results in a lowered utilization.

However, there are species differences in fat availability since castor oil may be largely utilized in the rabbit, guinea pig and sheep where it exerts no cathartic action (McCay and Paul, '38; Paul and McCay, '42). McCay and Paul ('38) have also shown that guinea pigs are especially sensitive to high melting fats.

We became interested in a study of the digestibility of rape-seed oil because of the report by Boer et al. ('47) that rats grew less favorably on a diet containing rape-seed oil than on a diet containing butter. This finding has been corroborated in our laboratory (Deuel et al., '48). Rape-seed oil had previously been shown to be the slowest absorbed of a number of natural fats tested (Deuel et al., '40) so it might be suspected that the digestibility would also be the lowest

[1] This was aided by a research grant from Swift and Co.

of the common liquid fats. Although rape-seed oil is not used for edible purposes to any great extent in the United States, the cold pressed oil is widely used in Europe and especially in India for food purposes. It is used in England by bakers for greasing the ends of loaves of bread to prevent their sticking together (Lewkowitsch, '21).

It has been known for a long time that fat is excreted in the feces not only as the triglyceride and fatty acid which are ether-soluble but also in the form of soaps which are insoluble in ether. However, little attention has been paid to this latter fraction and the extensive series of tests of fat digestibility in man reported by Langworthy ('23) were based only on the fecal fat fraction which was directly soluble in ether. In recent tests on rats (Augur et al., '47; Crockett and Deuel, '47), it was shown that considerable amounts of ingested fats may be excreted as soaps. In the case of a number of low-melting fats such as margarine, hydrogenated cottonseed oil [2], prime steam lard and bland lard, the soap fraction was quite low and its inclusion in the excreted fat did not appreciably alter the calculated values of the coefficient of digestibility since the correction for metabolic fat is correspondingly increased. On the other hand, in the case of hydrogenated lards melting at 61°C. as well as with several samples of hydrogenated cottonseed oil, the soap content greatly exceeded the neutral fat-fatty acid value in spite of the fact that the latter fraction was also usually considerably increased. However, in the tests on the hydrogenated lard melting at 55°C., the soap fraction was largely increased without any marked rise in the excretion of neutral fat and fatty acid. Cheng and Morehouse ('48) have shown that the proportion of fat excreted as soap is much higher when a calcium-magnesium rich salt mixture (Osborne-Mendel, '17) is included in the diet than on a control diet containing no added salts. It is thus apparent that an entirely false impression of the digestibility of fat may be gained if account is not taken of the soap fraction.

[2] Crisco.

EXPERIMENTAL AND RESULTS

The experimental procedure was similar to that used by Augur et al. ('47). The diets containing the rape-seed oil were fed over 10-day periods. This included a preliminary 2-day orientation period followed by an 8-day interval during which the food consumption was measured and the feces were collected for fat analysis. The diet had the following percentage composition: commercial casein, 18; glucose, 56; rape-seed oil, 15; Osborne-Mendel ('17) salt mixture, 7; yeast,[3] 1; liver extract,[4] 3. Food was given ad libitum during the tests. The animals used were adult female rats from our stock colony. Total glyceride, fatty acids and soaps in the stools were determined by diethyl ether extraction of the dried stool. Soaps in the feces were determined by first converting them to fatty acids by acidification of the dried ether-extracted residue of the stools with a minimum quantity of 50% sulfuric acid; the acid paste was then re-extracted with ether.

The calculations for digestibility were made in the usual way after correction for the metabolic fat. This correction was determined in experiments carried out on a diet similar to the above except that the fat was replaced by glucose. The correction for metabolic fats used in these tests (65 mg per gram dried stool) is slightly higher than the value found by Augur et al. ('47) which was 50.5 mg.

The crude rape-seed oil used in the tests was a commercial product.[5] It was a dark thick viscous oil having a free fatty acid content of 0.6%. The refined rape-seed oil was prepared from the crude sample by bleaching and deodorizing[6]; it had a light yellow color, a satisfactory odor and a free fatty acid content of 0.01%. The following constants were found for the crude and the refined rape-seed oils, respectively: saponification number, 166.8 and 167.7; iodine number, 101.5 and 96.3.

[3] Anheuser-Busch, strain G.

[4] Wilson, 1: 20.

[5] From the Pacific Vegetable Oil Co., San Francisco.

[6] We wish to thank Mr. R. H. Neal of The Best Foods, Inc., for refining the rape-seed oil.

Two series of tests were carried out on rape-seed oil. Since the first tests on the crude oil sample gave the unexpectedly low coefficient of digestibility, the experiments were repeated with the highly-refined product. Ten rats were used in each series of tests. The results are summarized in table 1. The values on a margarine fat from earlier tests are included for comparison.

TABLE 1

Summary table of digestibility of crude and refined rape-seed oil compared with margarine fat when fed to female rats in a diet at 15% level.

DATA SUMMARIZED	CONTROL TESTS	CRUDE RAPE-SEED OIL TESTS	REFINED RAPE-SEED OIL TESTS	MARGARINE FAT TESTS[1]
Number of rats	10	10	10	10
Average weight of rats, gm	170	175	160	214
Average gain, gm	—4	4	—1	—1
Fat in diet, %	0	15	15	15
Average food eaten, gm	69	80	52	72
Average fat ingested, gm	0	12.1	7.8	10.7
Average weight of dried stools, gm	3.83	6.47	3.43	4.40
Average fat excreted				
As neutral fat and fatty acids, gm	0.12	1.07	0.65	0.24
As soaps, gm	0.12	2.12	0.99	0.30
Total, gm	0.25	3.19	1.64	0.54
Total, corrected, gm[2]		2.77	1.42	0.31
Coefficient of digestibility		77	82	97
Standard error of mean[3]		1.6	1.5	0.4

[1] Data from paper of Augur et al. ('47).

[2] Corrected for metabolic fat by multiplying stool weight by 65 mg.

[3] Calculated by the formula $\sqrt{\Sigma d^2/n-1} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

DISCUSSION

Rape-seed oil is the first fat, liquid at ordinary temperatures, for which such a low coefficient of digestibility as 77 has been found. This value is only slightly improved with a highly refined rape-seed oil and therefore must be attributed to the fat itself rather than to impurities present in the crude product. These results are in line with earlier tests (Deuel et al., '40) where it was shown that the rate of absorption of

rape-seed oil from the intestine was the slowest of any of a number of natural fats.

The poor digestibility of rape-seed oil must be related to its composition. This fat consists of 40 to 50% of trierucin and not more than 5% of the glycerides of saturated acids, with the balance being made up of triolein and trilinolein (Hilditch, '40). The feature which distinguishes it from the other vegetable oils having high digestibilities is the high content of trierucin. It is apparent that this triglyceride is largely hydrolyzed in the gastrointestinal tract as the principal fat excretion is in the soap fraction. Therefore, it would seem logical to assume that the difficulty in digestibility was associated with the poor absorption of erucic acid.

The reason for the discrepancy between the tests on rats and the earlier results on man (Holmes, '18) is not immediately apparent. Holmes reported an average coefficient of digestibility of 98.8 on 4 subjects who consumed an average of 82 gm of rape-seed oil daily. It is possible that this variation may be ascribed to a species difference. Another possible explanation may be that had the values obtained by Holmes ('18) been corrected for the fat lost as soap, a much lower coefficient of digestibility would have been recorded. The answer to this question can only be obtained by new experiments on human subjects where a determination of the excretion of fat in the form of soaps is made. Because of the widespread use of rape-seed oil as a food, it would seem to be of considerable importance that such tests be made on man.

SUMMARY

Rape-seed oil has been found to have the lowest coefficient of digestibility of any fat, liquid at ordinary temperature, which has been investigated on rats. Crude rape-seed oil was digested to the extent of only 77% while the refined oil gave an average value of 82%. This apparently cannot be traced to a failure of lipolysis but is believed to be related to the poor absorbability of the erucic acid fraction.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

X. ON THE REPUTED GROWTH-PROMOTING ACTIVITY OF VACCENIC ACID [1]

HARRY J. DEUEL, JR., SAMUEL M. GREENBERG, EVELYN E. STRAUB, DOROTHY JUE, CHESTER M. GOODING AND CARL F. BROWN

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles, and Bayonne Laboratory, The Best Foods, Inc.

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In a recent paper (Boer, Jansen and Kentie, '47), data were presented in support of the view that summer butter possesses a growth-promoting activity other than vitamin A, which is not shared by winter butter or by the vegetable oils. The factor responsible for this effect was found in the unsaturated fatty acid fraction and was identified as vaccenic acid. In another report (Boer, Jansen, Kentie and Knol, '47) vaccenic acid, prepared from hydrogenated China wood oil, was tested under similar conditions and was shown to produce the growth-promoting effect. It was therefore concluded that the factor in summer butter responsible for this activity is vaccenic acid. The earlier experiments of Boer and Jansen ('41) which indicated a superiority of butter over olive oil in producing growth in rats were not confirmed by Euler and his coworkers ('42, '43), but Boer, Jansen and Kentie ('47) at-

[1] This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Prof. Anton J. Carlson of the University of Chicago, Prof. Arthur W. Thomas of Columbia University and Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments.

The authors wish to thank the Winthrop Chemical Co. for the crystalline vitamin D_3 and Merck and Co. for the α-tocopherol.

tribute this to the absence of vaccenic acid in the winter butter of Sweden.

In earlier studies from this laboratory (Deuel et al., '44a), no differences could be demonstrated in the growth rate of young rats when fed on diets complete in the known nutritional requirements irrespective of whether corn, cottonseed, olive, peanut or soybean oil, a margarine, or a summer butter were incorporated in the diet. Similar results were obtained by Zialciti and Mitchell ('44) and by Deuel and Movitt ('45) when the test animals were rats which had been prematurely weaned. Such animals had been previously reported as being especially sensitive to a lack of an essential factor present in butter although it was stated that this sensitivity was lost after 6 weeks if the animals were maintained on a stock diet (Schantz, Elvehjem and Hart, '40; Schantz, Boutwell, Elvehjem and Hart, '40). In later tests from the present laboratory (Deuel et al., '46) vegetable oils, a margarine or a butter were found to be equally satisfactory in the diet in protecting against undernutrition or in satisfying the dietary needs for augmented growth which could be produced by the injection of the growth hormone.

Euler at al. ('46) have reported as satisfactory growth on a margarine fat mixture as on butter in rats over a 700-day period. Superior fertility and lactation along with a somewhat lowered mortality resulted from the margarine diet. These authors reported that the total weight of all offspring at 30 days in series A from 25 females on the butter diet was 8508 gm, compared with 15,955 gm for a like number of females receiving the margarine diet, and 15,047 gm for the animals receiving the stock diet. In series B, similar results were obtained although the performance of both the butter and margarine groups was improved by the addition of vitamin E to the diet. The total weight of all offspring at 28 days of age was found to be the following for series B: butter diet, 5422 gm; margarine diet, 8186 gm; butter diet + vitamin E, 7613 gm; and margarine diet + vitamin E, 9494 gm. Deuel et al. ('44b) reported no differences in reproduction or lactation

where both of the parents had been raised on the several vegetable fats or a margarine as compared with the diet containing butter. Euler points out that our failure to demonstrate a superiority for the vegetable fats was because our tests were carried out over a shorter period and with standardized litters.

The vegetable oils employed in our earlier tests were those produced and used for dietary purposes in the largest amounts in the United States. These included corn, cottonseed, olive, peanut and soybean oils. On the other hand, Boer et al. have used rape-seed oil and olive oil for their comparison with butterfat, although peanut oil was used in some of the preliminary tests. It was noted that "growth on rape-seed oil and on olive oil not only diminished at an earlier time than on peanut oil, but also that the difference in growth between the animals receiving butter and the control rats was greater than when peanut oil was used."

The present tests were undertaken to determine (1) whether any augmentation in growth could be demonstrated when vaccenic acid was used as a supplement to a typical vegetable oil such as cottonseed, and (2) whether the inferior growth obtained with the rape-seed oil diets might be ascribed to a lowered food consumption by rats on such diets. Finally, the tests were designed to determine whether any growth-promoting activity could be demonstrated with vaccenic acid of high purity.

EXPERIMENTAL

Twenty litters of our stock rats (Wistar strain) were used with litter mates equally distributed between the 6 dietary groups. Each group consisted of 20 rats (10 males and 10 females except for group IV in which there were 11 males and 9 females). The rats were placed on the diets at 29 days of age. Food was given ad libitum. The reserve diets were kept in the refrigerator except at time of feeding. In addition, all rats received as a supplement a daily oral dose of 0.1 ml of the same fat as that present in the diet which they were receiving. This included vaccenic acid (groups II and VI) or China wood oil (group III) in solution. In order to avoid the

criticism that variations in growth could be attributed to the administration of the fat supplements, similar amounts of the particular fat under investigation were administered irrespective of whether or not it contained vaccenic acid or hydrogenated China wood oil.

The diets used were essentially the same as those of Boer, Jansen and Kentie ('47). The composition is given in table 1, while the constants for the fats used are summarized in table 2.

TABLE 1

Composition of the diets used.

DIET CONSTITUENT	GROUP					
	I	II	III	IV	V	VI
	%	%	%	%	%	%
Whole ground wheat	72	72	72	72	72	72
Commercial casein	5	5	5	5	5	5
Yeast [1]	10	10	10	10	10	10
Salt mixture (Osborne-Mendel, '17)	3	3	3	3	3	3
Rape-seed oil [2]	10	10	..	..	..	..
Rape-seed oil containing 3% hydrogenated China wood oil [2]	..	..	10	..	..	..
Butterfat [2,3]	..	..	..	10	..	..
Cottonseed oil [2,4]	..	..	..	..	10	10

Following supplements also given daily (6 times weekly) by mouth: I, 0.1 ml rape-seed oil; II, 0.1 ml rape-seed oil containing 10 mg vaccenic acid; III, 0.1 ml rape-seed oil containing 3% China wood oil; IV, 0.1 ml butterfat; V, 0.1 ml cottonseed oil; VI, 0.1 ml cottonseed oil containing 10 mg vaccenic acid.

[1] Anheuser-Busch, strain G.

[2] The following supplements were added to all oils per 100 gm oil: carotene (General Biochemicals, Inc.) 2.4 mg; fish liver oil containing 350,000 I.U. per gram, 19.2 mg; crystalline vitamin D_2, 0.05 mg; α-tocopherol, 36.0 mg; commercial butter flavor, 1.2 mg.

[3] Challenge Brand obtained locally.

[4] Wesson oil.

Following the unexpected finding that pure vaccenic acid is something less than a nutritional supplement in the diet of the rat, it occurred to us that this singular effect might have been due to the presence of detrimental quantities of lead in our product, the last traces of which are removed only by very

TABLE 2

Constants of the fats and vaccenic acid used in the tests.

FAT OR FATTY ACID	SAPONIFICATION NUMBER [1]	IODINE NUMBER (WIJS) [1]	NEUTRALIZATION EQUIV.	MELTING POINT	REFRACTIVE INDEX, ABBE 70° C.	MICROANALYSIS	
						Carbon	Hydrogen
				°C.		%	%
Vaccenic acid							
Theoretical or literature	....	89.9	282.3	Various 38–39 inc.	1.4432 [2]	76.51	12.10
First lot	....	88.4	283.2	38.4 [3]	1.4415	77.61 [4]	12.63 [4]
Second lot	....	87.4	282.8	38.8 [3]	1.4434	76.96 [4]	12.42 [4]
						76.34 [5]	12.04 [5]
Crude rape-seed oil	166.8	101.5					
Refined rape-seed oil	167.7	96.3					
Butterfat	228.6	33.2					
Cottonseed oil	193.7	111.1					
Hydrogenated China wood oil	184.7	77.5					

[1] We wish to thank Amber L. S. Cheng for determining the constants on the several fats.

[2] Boeseken et al. ('30).

[3] Mixed melting points were unchanged.

[4] Analyses at California Institute of Technology by the courtesy of Dr. A. J. Haagen-Smit.

[5] Analyses at Columbia University through the courtesy of Professor H. T. Beans.

thorough acid washing. However, quantitative spectrographic analyses indicated that this was not the case.

The crude rape-seed oil was a commercial product [2] that had been refined, bleached, and deodorized before being shipped to us for the tests. It had a light color, 0.01% of free fatty acids (compared with 0.6% in the crude oil) and only a slight taste.

Although the butter used in the tests was purchased on the local market, it was unquestionably a summer butter. Cattle in this area are on green feed most of the year. An analysis of the present butter sample for carotene gave a total value of 7.2 μg of yellow pigment per gram of butter. No artificial coloring was present. From the saponified butter sample, β-carotene was separated by chromatography; on extraction from the column, this amounted to 5.2 μg per gram of butter. It gave an absorption curve typical of β-carotene as determined spectrophotometrically. These values are in the normal range of carotene in commercial butters (Fraps and Kemmerer, '43) and exceed those of Holstein cows on summer feed (Deuel et al., '42). One must conclude that the butter used was a "summer butter."

RESULTS

The results on the 6 groups of rats are summarized in table 3. The gain in weight of all groups receiving rape-seed oil (I–III) is in most cases significantly lower than the increase in weight of the animals receiving butterfat (IV) or cottonseed oil (V). There is no evidence that the supplementation with vaccenic acid improves the growth of the rats receiving rape-seed oil (II) or of those which were on a basal diet containing cottonseed oil (VI). In fact, there would seem to be a depressing effect on growth in the male rats in both of these groups. The results with hydrogenated China wood oil are also negative as far as growth-promoting activity is con-

[2] Pacific Vegetable Oil Co., San Francisco. The sample was refined, bleached, and deodorized in the Bayonne Laboratories of the Best Foods, Inc., after which it was shipped to us for the tests. We wish to express our appreciation to Mr. N. G. Barradas of the San Francisco branch of The Best Foods, Inc. for obtaining this sample of crude rape-seed oil for us.

cerned. A statistical analysis of the data shows that the ratio of mean difference to standard error of mean difference exceeds the significant value of 2.5 where groups I, II or III (males) are compared with groups IV or V (males) but in no other case.

Because of the considerable variation in starting weights in the several groups, a recalculation was made for 8 male and 8 female rats in each group so that the average initial weights would have a spread of less than 2 gm. Selections of the rats to be dropped from consideration were based solely on their starting weights. Where the original level of body weight was too low, the 2 rats with lowest weights were dropped [males I, II, III, VI; female IV (1 rat only)]. Where the beginning weights were too high, the rats with the highest starting weights were dropped from consideration (male V; females I, II, III, VI). The 2 lowest weight rats and one with average weight were dropped from the males in group IV, while the 2 rats with lowest and highest weights at the start were deleted from group V (females). The gain in weight in this revised group in no way alters the picture.

The food consumption of the rats was determined over the 6-week period. The average weight of the food ingested and the efficiency of utilization are reported in table 3. A higher efficiency of utilization of the diets by the male rats as compared with the females confirms our earlier observations (Deuel et al., '44a). When the caloric values of the diets are corrected for the utilizable fat, little difference in efficiency of utilization of the diets is to be observed, using the data for the "selected group."

DISCUSSION

The present experiments demonstrate that whether the diet contains a vegetable oil such as cottonseed oil, or butterfat, the growth of the rats is identical. In the present tests, the other components of the basal diet were whole ground wheat, commercial casein, yeast, salt mixture, and a mixture of fat-soluble vitamins. These results also confirm our earlier re-

port (Deuel et al., '44a) where the basal diet consisted of skimmed milk powder and the fat (vegetable oils, a vegetable oil margarine or a butter) fortified with the fat-soluble vitamins.

The present tests also confirm the results of Boer, Jansen and Kentie ('47) which show a superior growth for butterfat as compared with rape-seed oil. However, the results on rape-seed oil are not typical of vegetable oils in general because cottonseed oil (present report) and corn, cottonseed, olive, peanut and soybean oils (Deuel et al., '44a) all give a growth response identical with that for butter.

There are several possible explanations for the atypical results with rape-seed oil. In the first place, the crude product has an especially unappetizing taste which would be expected to cause a lowering of the food consumption with a resulting decreased growth rate. Although no data on food consumption are available from the Boer et al. experiments, the food consumption values for the rape-seed groups in the present tests are somewhat lower with the males but not with the females, even where a highly refined product was used.

It would also appear that the rape-seed oil diets have a lower efficiency of utilization. This is probably to be traced to the unsatisfactory digestibility of the rape-seed oil. Although it has generally been considered that all fats melting at 50°C. or lower are utilized to an extent of 95% or more, Deuel, Cheng and Morehouse ('48) have found that the coefficient of digestibility for crude rape-seed oil in the rat is 77.2 while that of the refined oil is only 82.5. When corrections are made in calculating the caloric values of the diets for the digestibility of the fats, the differences in the efficiency of utilization between the rape-seed oil diets, on the one hand, and the butterfat and cottonseed oil diets, on the other, largely disappear. It would thus seem evident that the lower growth rate on the rape-seed oil diets is to be ascribed mostly to the poor digestibility of the rape-seed oil as compared with the practically complete digestibility of butterfat and cottonseed oil.

TABLE 3

Summary table of mean starting weights, weight

TABLE 2

Summary table of mean starting weights, weight gains, and food consumption of rats receiving a diet containing rape-seed oil (I), rape-seed oil + vaccenic acid (II), rape-seed oil + hydrogenated China wood oil (III), butterfat (IV), cottonseed oil (V), or cottonseed oil + vaccenic acid (VI) over a 6-week period.

DIET NO.	STARTING WT. AT 29 DAYS OF AGE			GAIN DURING 6 WEEKS			FOOD INTAKE IN 6 WEEKS			DIET EFFICIENCY [2]		
	M [1]	F [1]	Average	M [1]	F [1]	Average	M	F	Average	M	F	Average
	gm	gm	gm	gm	gm	gm	gm	gm	gm			
I	64.6 ± 3.2	66.4 ± 2.6	65.5	152.0 ± 8.3	91.3 ± 3.6	121.6	484.3	419.6	452.0	7.69	5.33	6.59
II	63.2 ± 3.2	66.4 ± 3.2	64.8	144.4 ± 7.0	91.4 ± 5.0	117.9	472.8	423.3	448.1	7.49	5.29	6.45
III	66.8 ± 2.3	63.2 ± 2.6	65.0	158.0 ± 3.5	92.3 ± 3.5	125.2	527.9	420.7	474.3	7.34	5.38	6.47
IV	65.5 ± 3.5	57.8 ± 2.4	61.7	183.9 ± 3.5	101.0 ± 3.5	142.5	540.2	408.6	474.3	8.34	6.06	7.36
V	71.7 ± 3.2	60.1 ± 3.2	65.9	178.6 ± 5.0	103.7 ± 6.0	141.2	554.5	409.1	481.8	7.90	6.22	7.18
VI	64.2 ± 1.8	68.3 ± 3.3	66.2	169.2 ± 5.4	101.5 ± 2.8	135.4	526.1	439.7	482.9	7.85	5.66	6.87
	Calculations based on 8 males and 8 females — "selected group"											
Ia	68.1 ± 2.7	63.5 ± 1.8	65.8	154.5 ± 6.0	92.4 ± 4.2	123.5	495.1	416.7	455.9	7.98	5.67	6.93
IIa	65.6 ± 3.5	63.1 ± 2.5	64.4	149.7 ± 6.0	92.7 ± 5.8	121.2	490.6	421.3	455.9	7.79	5.63	6.80
IIIa	69.3 ± 2.0	60.4 ± 2.0	64.9	158.1 ± 4.3	88.4 ± 4.0	123.2	529.5	401.1	465.3	7.64	5.63	6.78
IVa	69.0 ± 3.3	59.4 ± 1.3	64.2	186.6 ± 4.4	99.0 ± 3.3	142.8	554.5	409.1	481.8	8.31	5.97	7.32
Va	68.5 ± 2.6	60.1 ± 2.6	64.3	179.4 ± 5.7	105.8 ± 7.5	142.6	558.5	415.2	486.9	7.93	6.29	7.23
VIa	66.3 ± 1.5	64.4 ± 2.3	65.4	162.5 ± 2.8	101.0 ± 3.6	131.7	516.6	434.1	475.4	7.77	5.74	6.84

Each original group contained 20 rats (10 males, 10 females except group IV which had 11 males and 9 females).

[1] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$ where "d" is the deviation from the mean, and "n" is the number of observations. Because of wide variations in the gain of weight of males and females, the value would be meaningless for the combined groups.

[2] Ratio of $\frac{\text{gm gain} \times 100}{\text{Cal. consumed}}$. Calculated on basis of caloric values of the diets being 4.08 Calories per gram.

For the revised groups (Ia–VIa) the same ratio as above was used but based on following caloric values of diets:
Rape-seed oil diet — 3.91 Calories per gram (based on 82% digestibility of fat).
Butter and cottonseed oil diets — 4.05 Calories per gram (based on 97% digestibility of fats).

There is no evidence from the present tests of any improved growth when either purified vaccenic acid or hydrogenated China wood oil is added to the diet. In this observation, our results are at variance with those of Boer, Jansen and Kentie ('47) as well as Boer, Jansen, Kentie and Knol ('47). It is not known whether these investigators used a crude or a refined rape-seed oil. In the former case, a masking of the unappetizing flavor of the oil might have increased food consumption although there are no data in their reports on the amount of food consumed. No change in the food intake was noted in our tests where a refined rape-seed oil was used whether the diets were supplemented or not with vaccenic acid or hydrogenated China wood oil.

Vaccenic acid apparently cannot be identical with the factor which Schantz, Elvehjem and Hart ('40) have indicated as being present in butter. The latter factor was believed to be a long chain *saturated* acid while vaccenic acid belongs to the group of *unsaturated* acids. There was no indication that the content of the factor of Schantz et al. varied in summer and winter butter. Vaccenic acid is believed to be present only in summer butter. Finally, the need for the Schantz et al. factor was only apparent during the period immediately following weaning at 21 days of age; the depletion for the vaccenic acid tests was continued 3 or 4 weeks after weaning at 28 days, by which time Schantz and associates found that most of the differences between the various fats had disappeared.

The experiments of Jack et al. ('45) as well as those of Henry at al. ('45) indicate that there is no one fraction of butterfat the growth promoting action of which is superior to that of whole butter. However, Geyer et al. ('47a) have recently reported a superior growth-promoting action of the liquid fraction of butter remaining after the separation of the solid portion from an acetone solution at —4°C. They obtained positive results only on a single sample of September, 1945 butter but they were unable to confirm it on June or December, 1945 samples as well as on butters obtained during

February, June, July or September, 1946. Food consumption was not determined for the single positive test.

Although there would seem to be some evidence of the presence of vaccenic acid in butter and animal fats (Bertram, '28; Grossfeld and Simmer, '30; Geyer et al., '47b), and also in margarine (Bertram, '28; Grossfeld and Simmer, '30) as well as its absence in vegetable oils (Geyer et al., '47b), the evidence is somewhat weak in view of the fact that the $\triangle^{12,13}$-octadecenoic acid which melts at 39.5° and 39.7–40.1°C. (Richter, '42) has not been excluded. There is no indication from the present experiments that vaccenic acid itself possesses any specific function in growth.

SUMMARY

1. No differences in the rate of growth of male and female rats were noted over a 6-week period when the diet contained butterfat or cottonseed oil.

2. The growth rate of rats receiving rape-seed oil diets was somewhat less and the efficiency of utilization of these diets poorer than those obtained on the butter or cottonseed oil diets. It is believed that the less efficient utilization of the rape-seed oil diet is to be attributed to the poor digestibility of the fat which may be due to its characteristically high content of eracic acid ($\triangle^{13:14}$ docosenoic acid).

3. No stimulating effect on growth was produced by the administration of vaccenic acid or hydrogenated China wood oil to the rats on the rape-seed oil diet. Moreover, no increased growth resulted in the cottonseed oil group when a supplement of vaccenic acid was fed.

4. It is concluded that vaccenic acid plays no specific role in relation to growth of the rat.

APPENDIX

Preparation of vaccenic acid (Gooding and Brown[3]*).* Vaccenic acid was separated from partially hydrogenated China wood oil[4]

[3] Research Laboratories, The Best Foods, Inc.

[4] One sample of the crude China wood oil was obtained from the Standard Varnish Co., Staten Island, New York, and the second sample from Werner G. Smith Co., Elizabeth, New Jersey.

by the method of Boeseken et al. ('30) with some modifications. The acidity of the crude oil was reduced by esterification with methanol prior to hydrogenation. In order to minimize polymerization and wandering of double bonds, the hydrogenation to iodine values ranging from 80 to 86 was accomplished in less than 10 minutes by use of 1% commercial nickel catalyst at 45 p.s.i. and at 100° to 200°C. The hydrogenated glycerides were converted to methyl esters by alkaline alcoholysis and the esters distilled to remove polymers. The fatty acids from the distilled esters were treated with lead acetate and the lead soaps were recrystallized once using the proportions of reagents recommended by Hilditch ('40).

In general terms, pure vaccenic acid was separated from the fatty acids of the lead soaps by first removing the major part of saturated acids by crystallization from methanol at about 8°C. The filtrate (I) was diluted with 5 volumes per cent of water and again held at 8°C., whereupon the remaining saturated acids and a large part of the vaccenic acid were obtained as a filter cake (II). The filtrate from II was then held at 0°C. to obtain the first crop of crude vaccenic acid (III). The filtrate from III was repeatedly reused as a solvent for filter cake II to obtain successive crops of crude vaccenic acid by repeating the sequence of crystallizations and filtrations already described. The volume of solvent varied from 3 to 15 times the weight of acids and all filtrations were done in the refrigerator after at least 16 hours, using precooled Buchner funnels fitted with a rubber dam. The yields of crude vaccenic acid were combined and finally purified by repeating the described process or alternately by crystallizations from methanol. Two different lots of vaccenic acid were prepared and both used in the feeding tests. The identity of the material with vaccenic acid is indicated by the constants obtained, which are summarized in table 2.

The final proof of structure of our vaccenic acid was obtained by $KMnO_4$ oxidation of its methyl ester in acetone. The monocarboxylic acids resulting from the oxidation were found to be the expected heptanoic acid, together with the further degradation product, hexanoic acid (Markley, '47); they were redistilled and identified by their boiling points and neutralization equivalents. The dicarboxylic acid was found to have a neutralization equivalent of 109 (calculated for nonanedicarboxylic acid 108) and a melting point of 96°–100° which was lower than the reported 110°C. for this acid but which was in agreement with that found by Boeseken et al. ('30).

We believe that the slightly low iodine value for our material was in part due to the failure of the Wijs reagent, at least as we used it, since pure elaidic acid was also found to have an iodine value of 88.4 (theor. 89.9). Boeseken et al. ('30) reported 88.1 as an iodine value for vaccenic acid.

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THE GLYCINE REQUIREMENT OF YOUNG POULTS

F. H. KRATZER AND DELBERT WILLIAMS
Division of Poultry Husbandry, University of California, Davis

ONE FIGURE

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The need for glycine in the diet of the young chick has been shown by feeding rations in which the amino acids were largely supplied by casein (Almquist et al., '40; Almquist and Mecchi, '40, '42; Hegsted et al., '41), and by feeding rations containing mixtures of amino acids (Almquist and Grau, '44; Hegsted, '44). Jukes et al. ('47) observed that poults grew at a reduced rate when gelatin was replaced by casein in a purified ration in which casein was the only other source of protein, and suggested that the effect of gelatin was due to the glycine which it contained. However, Kratzer et al. ('47) found that casein contains less arginine than is required for the optimum growth of young poults, which suggests that at least some of the growth reduction observed in poults by Jukes et al. may have been due to the deficiency of arginine.

As part of a research into the amino acid requirements of poults, the dietary requirement of young turkeys was investigated by use of a purified ration in which the amino acids were supplied by casein, arginine, cystine and varying levels of glycine.

EXPERIMENTAL

Day-old poults were fed a practical starting ration for about 6 to 8 days before they were given an adequate purified ration

to accustom them to such a diet. The adequate purified ration contained dried brewers' yeast, casein, gelatin, bone meal, salt, choline chloride, fish oil, soybean oil and cornstarch. After being fed the purified ration for 3 or 4 days the poults were divided into groups of 6 or 7, on the basis of body weight, and were fed the experimental rations. The poults were housed in electrically heated batteries with wire floors. The trials were continued for from 10 to 16 days.

The basal ration contained the following ingredients in grams: washed casein, 28; calcium gluconate, 5; cellulose [1], 5; salt mixture, 5; crude soybean oil, 3; tri-calcium phosphate, 2; vitamin mixture, 2; dicalcium phosphate, 1.5; L-arginine monohydrochloride, 0.6; fish oil (1000 A–400 D), 0.5; choline chloride, 0.3; L-cystine, 0.2; inositol, 0.1; cholic acid, 0.1; and sufficient cornstarch to make a total of 100 gm. The vitamin mixture had the following composition in milligrams: thiamine, 0.5; riboflavin, 0.5; pyridoxine, 0.4; calcium pantothenate, 1; 2-methyl-1,4-naphthoquinone, 1; niacin, 10; dl-alpha tocopherol acetate, 1; pteroylglutamic acid,[2] 0.1; and biotin,[3] 0.01. The composition of the salt mixture in grams was: sodium chloride, 1; dipotassium phosphate, 0.5; magnesium sulfate, 0.3; potassium chloride, 0.3; sodium silicate, 0.2; manganese sulfate, 0.03; aluminum sulfate, 0.025; ferric oxide, 0.02; copper sulfate, 0.005; zinc sulfate, 0.005; cobalt acetate, 0.002; and potassium iodide, 0.001. Glycine was added at the expense of starch.

Four experiments were completed in which the levels of added glycine varied up to 2% and there were 7 groups which were unsupplemented. Only 3 poults died in all the tests; these losses were apparently not due to the diets.

[1] Cellu flour.

[2] The pteroylglutamic acid was kindly supplied by Lederle Laboratories Inc., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

[3] Synthetic biotin was kindly supplied by Merck and Company, Rahway, N. J., through the courtesy of Dr. D. F. Green.

RESULTS AND DISCUSSION

The results of the 4 experiments are shown in figure 1. The rates of growth expressed as per cent gain per day [4] varied with the level of added glycine in the ration. As a basis for comparison, the rates of gain of poults fed stock mash in experiments 2, 3 and 4 were 6.9, 7.3 and 6.1% gain per day, respectively.

The growth of poults in general increased slightly as the level of glycine increased until approximately 0.75% glycine was fed. As the level of glycine increased beyond 1.5%, the gains in weight showed a gradual decline. It is evident from the averages shown in figure 1 that the poults in different trials grew at different rates. This was probably due to differences in the poults from different hatches and to slight variations in environmental conditions which we were not able to control. Poults and conditions within a given experiment were comparable.

When the various trials are considered, it is at once apparent that there were no differences between poults fed 0.75 to 1.25% of added glycine, since the average differences in the 10-day gains varied from 5.1 gm (trial 4) to 6.1 gm (trial 2). If we now pair the average gains of groups of poults fed the unsupplemented basal ration with the gains of groups fed 0.75 to 1.25% added glycine in the same experiment, 7 pairs of unsupplemented versus supplemented groups result. The average difference in such a comparison was found to be 19.4 gm giving a value for t of 7.638 which is statistically highly significant ($t = 3.707$, $p = 0.01$, Fisher, '46). There can be no doubt, therefore, that the addition of 0.75 to 1.25% glycine increased the rate of growth.

Although glycine deficiency caused a reduction in growth, the magnitude of the reduction was only slight. This is shown by the fact that the groups receiving no added glycine (fig. 1)

[4] Per cent gain per day $= \frac{\text{Total gain} \times 100}{\text{Average weight} \times \text{number of days}}$.

grew at a rate comparable to those receiving the stock ration (see above). These results suggest that the comparatively large reduction in growth observed by Jukes et al. ('47) when casein was the only source of protein was most probably due to a deficiency of arginine as well as of glycine, since a deficiency of glycine alone would be expected to produce only a slight decrease.

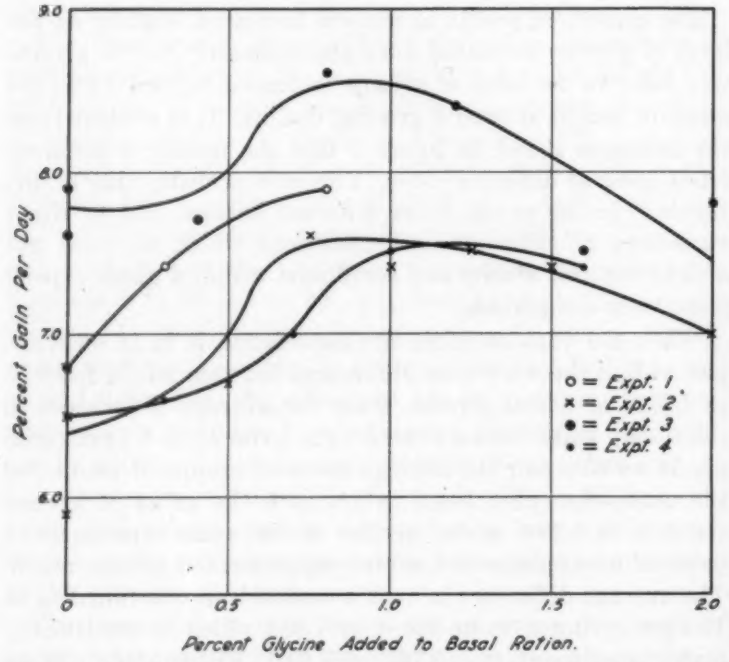

Fig. 1 The relation of the growth of poults to the amount of glycine added to the ration.

Almquist and Mecchi ('42) observed that the growth of chicks was somewhat reduced as the amount of glycine in the ration increased from 1.5 to 2.0%. Patton and Palmer ('36) also noted that glycine fed to laying hens at a rate of 4 gm per day was extremely detrimental to the birds. As

shown in figure 1, the growth of young poults declined as the amount of glycine added to the ration increased above approximately 1.5%. The results of the work on the 2 species indicate that both chicks and poults do not tolerate levels of glycine much in excess of the level which permits optimal growth.

According to Block and Bolling ('45) casein contains approximately 0.5% glycine, hence the basal ration contained 0.12% glycine. The 0.12% plus the 0.75% added glycine needed for optimum growth would place the glycine requirement of the poult at 0.87 or about 0.9% of the ration, a value which is slightly lower than the chick requirement of 1% when the glycine was in combined form, but considerably lower than the level of 1.5% found to be needed by chicks (Almquist and Mecchi, '42) when the glycine was supplied in pure form. The fact that growth of poults in these experiments was depressed less by a deficiency of glycine than was observed in chicks by Almquist and Mecchi ('42) also suggests that poults do not require as much dietary glycine as chicks. The evidence thus suggests that young turkeys possess a greater ability to synthesize glycine than do young chicks.

The lower dietary requirement for glycine of poults as compared with chicks is of special significance in view of the fact that poults require more lysine (Grau et al., '46) and arginine (Kratzer et al., '47), as well as more total protein, than chicks. Evidently the higher protein requirement of poults is not reflected in a higher requirement for all of the indispensable amino acids.

SUMMARY

Young poults which were fed a glycine-low ration in which the amino acids were supplied by casein, arginine and cystine grew at a rate comparable to that of poults fed a practical starting ration. Growth was slightly improved by the addition of glycine. Approximately 0.9% of total glycine was the smallest amount which produced optimal growth.

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GROWTH AND REPRODUCTION OF SWINE ON A PURIFIED DIET[1,2,3]

WALTER C. RUSSELL, ARTHUR E. TEERI[4] AND KLAUS UNNA[5]

Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey, and Merck Institute for Therapeutic Research, Rahway, New Jersey

ONE FIGURE

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Growth of swine on purified diets has been reported by several groups of investigators whose experimental objective was the study of vitamin deficiencies. Recently, McRoberts and Hogan ('44) reported on the adequacy of simplified diets and referred to a number of earlier studies of this type.

The experiments presented in this communication were designed to study the effects of a long period of feeding of a purified diet on the development and reproduction of swine.

EXPERIMENTAL

Pigs of the Jersey-Duroc strain, weaned at 28–31 days, were used. Litter A, born in September, consisted of 6 animals (nos. 25 to 30) and litter B, born in April, comprised 10

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[4] Present address, Department of Agricultural Chemistry, University of New Hampshire, Durham, New Hampshire.

[5] Present address, Department of Pharmacology, University of Illinois Medical School, Chicago, Illinois.

animals (nos. 66 to 75, inclusive). They were distributed in 3 groups, as shown in table 1 and fed as described below.

Group 1, consisting of 2 boars and 3 sows, was fed a purified ration having the following percentage composition: casein,[6] 25; dextrose,[7] 54; hydrogenated cottonseed oil,[8] 13; salt mixture no. 1, U.S.P. XI, 4; bone ash, 2; cod liver oil, 2; and choline chloride, 0.1. The protein content was 20 to 21%.

The members of the vitamin B-complex, available in crystalline form, were compounded in capsules. These were administered daily (except Sunday) by means of a capsule gun which released the capsule in the pharynx. Each capsule contained 2 mg thiamine, 4 mg riboflavin, 20 mg nicotinic acid, 20 mg Ca pantothenate, 20 mg inositol, 10 mg p-aminobenzoic acid and 2 mg pyridoxine. After a body weight of 100 lbs. was reached, the daily dose of these vitamins was doubled. All pigs were given 3 mg alpha-tocopherol by capsule, twice daily. The dosage of the daily vitamin supplement was adopted with the intention of providing the pigs amply with all crystalline B-vitamins. The dosage used, however, was rather arbitrary, since the requirement of pigs has not been established for some of these factors (inositol, p-aminobenzoic acid) nor has it been quantitatively determined for others (nicotinic acid, Ca pantothenate and pyridoxine). The amount of thiamine necessary for maximum growth of pigs maintained on purified diets similar to ours, has been determined as being between 125 and 141 μg per 100 gm of carbohydrate and protein (Ellis and Madsen, '44), and the daily requirement for riboflavin appears to be between 1 and 5 mg per 100 lbs. of body weight (Hughes, '40).

For group 2 (3 boars and 1 sow) the same dietary regime was followed as for group 1, except that each animal was fed 10 gm of dried whole liver daily. The dried powder was placed on top of the basal ration and was readily consumed. The ad-

[6] Vitamin-free (Harris).

[7] Cerelose.

[8] Crisco.

TABLE 1

Weight gains and food consumption.

FEEDING REGIME	LITTER, ANIMAL NO. AND SEX	BODY WEIGHT		AFTER 255 DAYS ON EXPERIMENT [1]			TOTAL DAYS ON EXPERIMENT	FINAL WEIGHT
		Init.	After 140 days on experiment	Weight	Av. daily food consumption	Gain per lb. protein consumed		
		lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*		*lbs.*
Group 1, purified diet, crystalline vitamins, 21% protein	A 25 M	15	110	223	2.5	1.56	469	288
	B 66 M	10	154	271	3.3	1.50	294	273
	A 26 F	11	96	201	2.4	1.50	469	338
	B 67 F	13	159 [2]					
	B 68 F	15	160	276	3.5	1.41	294	288
Group 2,[3] purified diet, crystalline vitamins, dried liver, 21% protein	A 27 M	14	137	257	3.1	1.47	394	350
	B 69 M	11	166	265	3.9	1.22	294	272
	B 70 M	9	147 [2]					
	B 72 F	12	161	274	2.8	1.75	294	303
Group 3, "farm ration," 17% protein	A 29 M	12	159	351	4.9	1.60	469	430
	B 73 M	12	151	315	4.7	1.60	294	367
	B 74 M	9	104 [2]					
	A 30 F	13	145	275	4.6	1.29	469	375
	B 75 F	11	127	260	3.5	1.33	294	303

[1] 283 days of age.

[2] Sacrificed for histological studies after 140 days of experiment.

[3] In group 2, no. 28 died after 74 days on experiment, and no. 71 was sacrificed for histological studies after 106 days on experiment.

dition of the liver raised the protein content of the ration not more than 1%.

Group 3 served as a control for the animals maintained on the purified diet. It consisted of 3 boars and 2 sows which were fed a hog feed of the open formula type, referred to as the "farm ration." The feed had the following percentage composition: yellow corn, 50; flour middlings, 29; meat scrap (55% protein), 9; soybean oil meal (41% protein), 5; low fiber alfalfa meal, 5; ground limestone, 1.5; and sodium chloride, 0.5. The guaranteed analysis was minimum protein, 17.0%; minimum fat, 3.5%; and maximum fiber, 6.0%. Vitamin D was not supplied because there was sufficient exposure to sunlight.

RESULTS

Growth

The distribution and disposition of the animals, along with food consumption, are shown in table 1; the growth response is shown in this table and in figure 1. After 140 days of experiment, the animals of litter A fed the "farm ration" showed better gains than their litter mates which received the purified diet with crystalline vitamins. On the diet with the additional supplement of dried liver, the growth response fell between those of the other 2 groups. On the other hand, the animals of litter B on the purified diets grew better than those of litter A and, especially those which received the dried liver supplement, better than those on the "farm ration."

During the first 255 days of the feeding trial (i.e., 283 days of age), all of the animals were housed and fed under comparable conditions. In litter A at the end of this period, boar 29 on the "farm ration" was significantly heavier than boar 27 on the purified diet with crystalline vitamins and the dried liver supplement, and both animals were markedly heavier than boar 25, which received the purified ration and crystalline vitamins. Also, sow 30 on the "farm ration," showed a greater weight than sow 26 on the purified diet with synthetic vitamins. At the end of the 255-day feeding period, boar 73 of

litter B which received the "farm ration," was heavier than litter-mate boars in groups 1 and 2. The sow of litter B on the "farm ration" (no. 75) was slightly lighter in weight than her litter mates on the supplemented purified ration (nos.

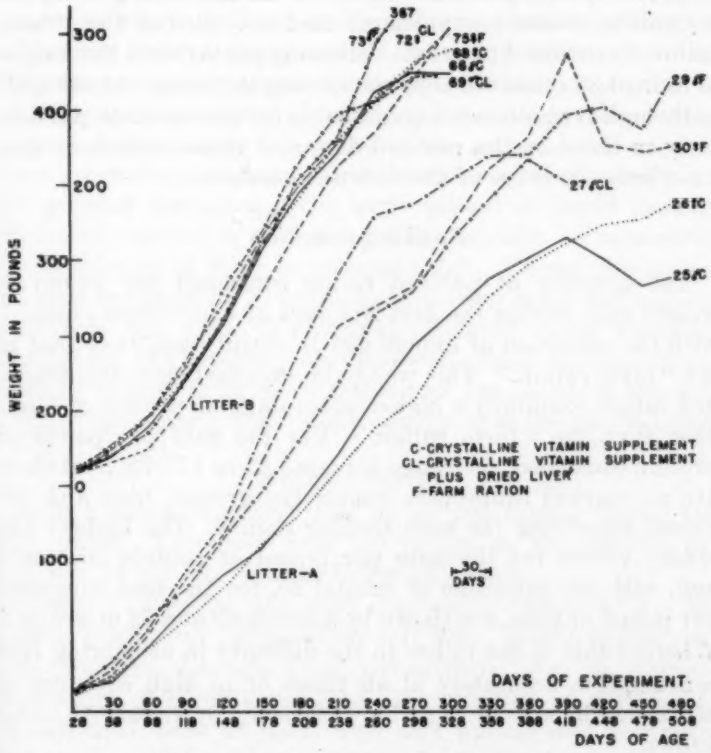

Fig. 1 Growth of swine on the supplemented purified diet and on the "farm ration."

68 and 72) receiving crystalline vitamins and crystalline vitamins and dried liver, respectively, which latter animals had essentially the same body weight.

Except for boar 27, the animals of litter A were continued on the experiment for 214 days longer until they were 497

days of age (fig. 1). In the period following the first 255 days on the experiment, animal 27, receiving liver supplement, and no. 25, receiving only the crystalline vitamin supplement, began to lose weight after 364 and 375 days on the experiment, respectively. On the other hand, no. 26 that was getting the crystalline vitamin supplement and no. 29, fed the "farm ration," continued to grow. Following parturition, the weight of animal 30 remained constant during lactation. At autopsy, pathological conditions were found in certain animals, particularly in those on the purified diet, and these conditions may have been the cause of the decline in weight.

Food consumption

The quantity of purified ration consumed per pound of weight gain during the first 255 days of experiment (table 1, with the exception of animal 69) is slightly less than that of the "farm ration." This was to be expected since the simplified ration contained a higher percentage of protein and less fiber than the "farm ration." For the gain per pound of protein consumed, the range is from 1.22 to 1.75 lbs., but there are no marked differences among the groups, high and low values appearing for each feeding regime. The highest and lowest values for the gain per pound of protein consumed and, with the exception of animal 30, for the feed consumed per pound of gain, are shown by animals 69 and 72 of group 2. Whether this is due either to the difficulty in measuring feed consumption accurately at all times or to high efficiency of utilization by animal 72 and low efficiency by animal 69, is not known.

Appearance of animals

Daily examinations were made, special attention being given to the character of the feces, condition of coat, gait, and condition of the eyes. In the early weeks of the experiment, the feces of all pigs of litter A, even of those on the "farm ration," were soft, and at frequent intervals even diarrheal in character. As the experiment progressed, the feces became well-

formed and only at infrequent intervals were they soft in the case of the animals on the purified diets, and rarely so with the animals on the "farm ration." During the first few months a number of the animals walked with a stiff, jerky gait, at times on tiptoe, or in a fashion resembling goose stepping. Abnormalities in gait appeared earlier and more markedly in animals of litter A than in those of litter B. The animals on the "farm ration" showed lesser signs of abnormal gait. In all animals, however, the abnormal gait gradually disappeared after 6 to 10 months. In general, the coats of the animals were normal although somewhat thin in several animals on the purified diet during the early period of rapid growth. Their hair was often matted together, probably on account of the scattering of their hygroscopic diet. Except for an occasional conjunctival exudate, no abnormal condition was observed in the eyes. All animals behaved quite normally throughout the experiments. However, those on the "farm ration" seemed to be more active and presented, in general, a better appearance than their litter mates on the purified diet.

Autopsy findings

Only 1 animal (no. 28) died of natural causes during the experiment. Autopsies were performed on all animals except nos. 27 and 74, with the following results:

Group 1. Purified diet with crystalline vitamins. No. 25, chronic pneumonia of the entire left lung. Bilateral obliterative pleuritis. Chronic focal pericarditis. *No. 66,* chronic pneumonia of right lower lobe. *No. 26,* foci of pericardial fibrosis near base of heart over left auricle and ventricle. *No. 67,* lower lobes of both lungs showed red consolidation. Remainder of lungs normal. Most of small intestine had pinpoint-sized red spots. Ulcers varying from 1 mm to about 8 mm in diameter were present in the cecum and scattered along the colon. Some were slightly raised; others extended about 1.5 mm into the lumen and had umbilicated centers. The mucosa of the colon had a granular appearance. *No. 68,* no pathological findings.

Group 2. Purified diet with crystalline vitamins and dried liver. No. 69, no pathological findings. *No. 70,* some fatty infiltration in the marrow of the femur. *No. 71,* no pathological findings. *No. 72,* some pleural adhesions on the left side. Marked adhesive pericarditis, mainly at the base of the heart.

Group 3. "Farm ration." No. 29, no pathological findings. *No. 73,* no pathological findings, except for dark red color and flabby consistence of the spleen. *No. 30,* no pathological findings. *No. 75,* no pathological findings. Pregnant with 6 fetuses.

Of 9 animals maintained on the purified diet, only 3 presented a normal picture on gross examination at autopsy. Evidence of infection was found in 4 animals receiving the supplement of crystalline vitamins, and in 1 receiving the additional supplement of liver. On the other hand, none of the animals maintained on the "farm ration" showed pathological tissue changes, with the exception of a dark and flabby spleen in 1 case.

Therefore, nutrient factors other than those supplied to groups 1 and 2 are apparently necessary to maintain swine in a normal condition on a purified type of diet, in spite of the satisfactory growth performance of some of these animals.

MATING AND REPRODUCTION

On the purified diet with synthetic vitamins

Boar 25 and sow 26. Attempts to mate these animals were begun early in July when the animals were 280 days old and had been on the experimental diet for 252 days. On many occasions the boar showed no interest, while at other times he became excited and made futile but short-lasting attempts to service the sow. Finally service was accomplished, but implantation did not take place, since at autopsy 83 days later, fetuses had not formed.

Boar 66 and sow 68. Mating attempts were begun at 200 days of age, after the animals had been on the experimental diet for 170 days. Behavior was essentially the same as that of

animals 25 and 26, but mating was not accomplished. Sow 68 was serviced, however, by boar 73 which had been fed the "farm ration." At autopsy, 32 days later, there was no evidence of implantation.

On the purified diet with synthetic vitamins and dried liver

Boar 27. A sow was not available on this diet. Attempts to mate this animal with sow 26 and with sows maintained on a "farm ration" and pasture were unsuccessful.

Boar 69 and sow 72. Attempts to mate these animals were not successful, but sow 72 was serviced by boar 73 of the group which received the "farm ration." At autopsy, 25 days later, there was no sign of implantation.

On the "farm ration"

Boar 29 and sow 30. These animals were placed on pasture, with free access to the "farm ration," when they were 283 days of age and after they had been on the "farm ration" for 255 days. On November 23, sow 30 farrowed 7 normal pigs and nursed them to weaning at 45 days of age. Therefore, the mating of these animals must have occurred about August 1 or 2, approximately 3 weeks after they were allowed access to pasture.

Boar 73 and sow 75. In contrast with animals 29 and 30, these animals did not have access to pasture and received only the "farm ration." They were mated without difficulty when 242 days of age, and 83 days later, at autopsy, the sow was found to be pregnant with 6 fetuses.

DISCUSSION

The question whether a purified diet similar to that used in our experiments and supplemented with crystalline vitamins meets the nutritional requirements of growing pigs has been studied in several laboratories. Wintrobe, Miller, Follis et al. ('42) placed 3 pigs 88 days old on a purified diet supple-

mented with thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline. Their animals developed normally and during the test period of 148 days grew at a rate which was only slightly inferior to that obtained on an adequate diet of crude food stuffs. In a later study, the same group of authors (Wintrobe, Stein, Follis and Humphreys, '45) repeated their experiment on 2 pigs 16–22 days old for a period of 120 to 203 days with similar results, although the vitamin supplement lacked niacin but included inositol. Apparently, the experimental period in both studies was too short to allow observations on reproduction.

McRoberts and Hogan ('44) failed to obtain satisfactory growth when they placed pigs at the age of 2 days on a purified diet supplemented with all vitamins available in crystalline form. On the other hand, the addition of relatively large amounts of various extracts from yeast or liver (5% in the diet) enabled the piglets to grow at a satisfactory rate during the experimental period of 20 to 54 days. It is evident from these experiments that pigs — at least during the period of weaning — require nutritional factors other than those provided by the supplement of crystalline vitamins.

The pigs in the present study were placed on a purified diet after weaning at 28 to 31 days of age. The results obtained were comparable to those of Wintrobe, Miller, Follis et al. ('42) and Wintrobe, Stein, Follis and Humphreys ('45), in that satisfactory growth and apparently normal development were obtained on a purified diet supplemented with crystalline vitamins. During the first 3 months of the experiment both sexes grew at least as rapidly on the purified diets as their litter mates on an adequate diet of crude food stuffs. Thereafter, the boars on the "farm ration" gained more weight than those on the purified diet (nos. 29 and 73 vs. nos. 25, 27, 66 and 69). Growth of the sows maintained on the purified diet was, with the exception of no. 26, equal to that obtained for the sows on the "farm ration" over a period of 255 days (cf. nos. 68, 72 and 75). Furthermore, the addition of dried

liver to the purified diet resulted in an improved growth of 1 animal in litter A, but had no effect on the 2 animals in litter B.

At the time this experiment was conducted, biotin was not available in sufficient quantities in crystalline form, and folic acid had not been isolated. According to Cunha, Lindley and Ensminger ('46), a deficiency of biotin in swine results in alopecia, spasticity of the hind legs together with cracks in the feet and dermatosis, but these signs were not noted in the present study. When Lindley and Cunha ('46) fed a purified diet supplemented with crystalline vitamins, except biotin, deficiency symptoms did not appear unless sulfaphthalidine was incorporated in the ration. Therefore, it would appear that the pig obtains its supply of biotin from the intestinal tract in amounts sufficient to prevent the occurrence of these deficiency symptoms. The addition of dried liver to the ration of group 2 of our animals may have provided some biotin and also folic acid, but if present, these factors did not consistently improve growth.

A deficiency disease due to lack of folic acid has not been described in pigs. Signs of deficiency which might be the result of the lack of folic acid in the purified diet could not be noted in this study. However, it remains to be decided whether lack of biotin, or folic acid, or both is responsible for both the increased susceptibility of the animals to infection and their failure in reproduction.

SUMMARY

Weanling pigs of 2 litters were maintained, for periods extending to 469 days, (a) on a purified diet supplemented with thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, p-aminobenzoic acid, and choline; (b) on the purified diet with an additional daily supplement of 10 gm of dried liver; and (c) on an adequate commercial hog feed ("farm ration" control).

1. During the first 3 months after weaning, growth on the purified diet was at least equal to that on the "farm ration."

Later, boars on the "farm ration" gained more weight than their litter mates on the purified diet.

2. Appearance and behavior of the animals maintained on the purified diet did not differ appreciably from that of the controls.

3. Feeding of the purified diet resulted in failure to reproduce.

4. The additional feeding of dried liver had no consistent effect on growth of the pigs maintained on the purified diet, and did not induce reproduction.

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PRODUCTION OF FUNCTIONAL AND FATTY CHANGES IN THE LIVER BY A CHRONIC VITAMIN B COMPLEX DEFICIENCY, AND INTERRELATIONSHIPS WITH PROTEIN INTAKE [1]

VICTOR A. DRILL AND TED. A. LOOMIS
Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

FOURTEEN FIGURES

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At present experimental and clinical studies of liver damage are centered chiefly around the effect of methionine and protein intake on the liver. Less attention has been focused on a possible effect of the vitamin B complex. The B vitamins first came into prominence, with respect to liver damage, when they were used in the treatment of alcoholic cirrhosis of the liver (Patek, '37). Since then a number of experimental studies have reported that a deficiency of the vitamin B complex can produce histological liver damage in rats and rabbits (McHenry and Patterson, '44; György, '44). In later work, however, it has been reported that the intake of protein and methionine plays the more important role in experimental hepatic injury. These studies, most of which were histological in character and performed on rats, have been reviewed recently (McHenry and Patterson, '44; György, '44). Of special interest is the report of hepatic cirrhosis in dogs, produced by feeding diets both low in pro-

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tein and deficient in one or more of the B vitamins (Fouts, '43).

Few experimental studies have been concerned with the relationship of the vitamin B complex to changes in liver function. Such studies of the vitamin B complex with respect to liver function have been reported in dogs fed a black-tongue producing diet (Rhoads and Miller, '38) and a ration free of vitamin B complex (Drill, Shaffer and Leathem, '43). Similar studies have been made during experimental hyperthyroidism (Drill and Hays, '42; Drill, Shaffer, and Overman, '43). In the studies on the vitamin B complex and thyroid feeding the changes in liver function generally followed the change in caloric intake. Nevertheless, the other studies (Fouts, '43; Rhoads and Miller, '38; Drill, Shaffer and Leathem, '43) lack adequate controls for inanition, so that one cannot judge whether the vitamin deficiency, the concomitant inanition, or both, were factors in producing the hepatic changes. Indeed, mere removal of protein from the diet of dogs will result in dye retention (Hough et al., '43).

The present study was designed to permit observations of possible interrelations between the vitamin B complex and protein as they affect the liver. A *chronic* deficiency of the *whole* vitamin B complex was produced under conditions of controlled protein intake. A *chronic* deficiency was chosen, rather than complete removal of the vitamin B complex from the diet, as being closer to conditions that might be observed clinically. Supplements of whole yeast, analyzed for various B vitamins, were used rather than synthetic vitamin mixtures, because the whole yeast contains more different B vitamins than are usually given in a synthetic vitamin mixture, and may also contain unknown factors. Since clinical recognition of changes in liver function will depend on functional tests and liver biopsies, these techniques rather than determination of hepatic lipids were used to study such changes. The effect of the chronic vitamin B complex deficiency was studied at 2 levels of protein intake and with adequate control of inanition.

METHODS

Adult mongrel dogs received a synthetic diet containing either 41% or 20% casein.[2] The 41% casein diet is considered to be a high protein diet, whereas the 20% casein ration is regarded as a normal, adequate protein diet. The dogs were allowed the diet *ad libitum* for a 3-hour period each day, after which the food intake was measured. The average food intake per week was calculated as calories consumed per square meter of body surface area per hour, using the formula of Cowgill and Drabkin ('27) for estimating surface area.

In each experiment the dogs were divided into 3 groups. Group I consisted of normal control animals that were used to measure normal fluctuations in food intake. Group II consisted of inanition-control dogs that were limited to the same number of calories, per square meter of surface area, as were consumed by the yeast-deficient dogs during the previous week. The dogs in groups I and II received a supplement of 4 gm of type 3 yeast extract per day,[3] which supplies a normal amount of the B vitamins. Group III consisted of chronic vitamin B-complex deficient dogs receiving a daily supplement of yeast no. 2019.[4] This analyzed yeast was weaker in vitamin content than the type 3 yeast extract, but was of sufficient potency so that the usual supplements fed each day could be weighed accurately. These dogs received 0.1 gm of yeast 2019 per kilo of body weight per day. In a trial experiment with 8 dogs, the chronically yeast-deficient animals were fed 0.2 gm of yeast 2019 per kilo per day. However, this amount of yeast fed over a 25-week period produced only a slight decrease in food intake and no decline in body weight,

[2] The 41% casein diet had the following percentage composition: casein, 41; sucrose, 33.6; lard, 21.5; bone ash, 2.6; and salt mixture (Karr, '20), 1.3, plus 20 drops of Oleum percomorphum per kilo of diet. In the 20% casein diet the sucrose was increased to 54.6%, thus keeping the amount of fat constant.

[3] Type 3 yeast extract contained: thiamine, 900 μg; riboflavin, 210 μg; pantothenic acid, 450 μg; nicotinic acid, 4 mg; and pyridoxine, 150 μg per gram.

[4] Yeast no. 2019 contained: thiamine, 18–30 μg; riboflavin, 65 μg; pantothenic acid, 150 μg; nicotinic acid, 550 μg; pyridoxine, 40 μg; choline, 4 mg; inositol, 4 mg; and biotin, 2.5 μg per gram.

and liver function and hepatic histology in these animals remained normal. A lower dose of yeast 2019 (0.1 gm per kilo per day) did produce a gradual decline in food intake and was used in the present studies. All dogs received 4 gm of type 3 yeast extract per day during the control period of the study when food intake and measurements of liver function were made.

Changes in hepatic function were studied by means of the bromsulphalein and serum phosphatase tests (Rosenthal and White, '25; Bodansky, '37). A blood sample was taken 30 minutes after the injection of 5 mg of dye per kilo of body weight. The concentration of dye present in the sample was divided by 2½, to return it to the scale of standards devised for the original 2 mg dose. A dye retention greater than 6% and serum phosphatase values above 5 units were considered abnormal. Liver biopsies and terminal liver sections were fixed in Bouin's solution and stained in hematoxylin-eosin. Tissue fixed in 10% formalin was stained for fat with Sudan III.

RESULTS

Experiment 1

High protein diet (41% casein) with minimal yeast supplement

All animals received the 41% casein diet and, following a control period of 5 weeks, 3 dogs were fed 0.1 gm of yeast 2019 per kilo per day (group 3) to produce a chronic deficiency of the vitamin B complex. Three normal animals (group 1) and 2 inanition controls (group 2) continued to receive 4 gm of type 3 yeast per day.

The dogs receiving the low intake of B vitamins showed a decrease in food intake during the fourth week of the study. By the twenty-fifth week the food intake was only one-third that of the normal dogs (fig. 1). Of the deficient animals, dog no. 1 died during the fourteenth week and dog no. 2 during the thirty-ninth week of the experiment. No change in liver function or histology was noted in the dogs. Renal lesions were

absent in the first dog, but the animal dying during the thirty-ninth week showed kidney lesions in the glomeruli and the tubules. The epithelial cells of the tuft and Bowman's capsule were swollen; proliferation with crescent formation was present; and there was exudate in the glomerular space. In the

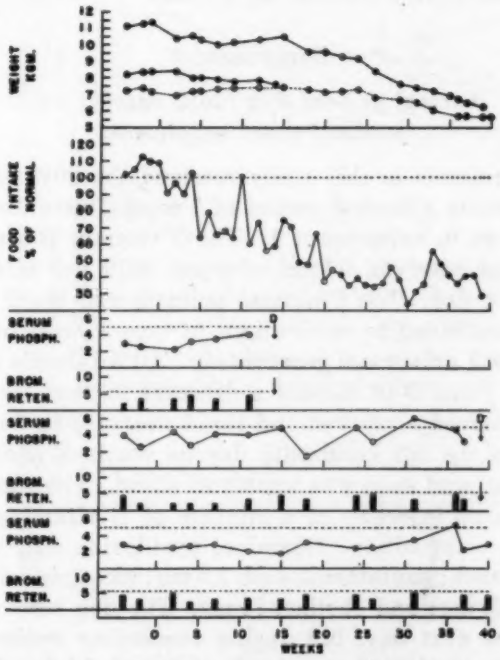

Fig. 1 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake, with a high casein (41%) diet. The food intake represents the average of the 3 dogs receiving 0.1 gm/kg/day of yeast 2019. The liver function tests for dogs 1, 2 and 3 are shown in a descending order. Compare with figures 2, 3 and 4, with dogs receiving the same yeast intake, but a normal (20%) casein diet.

proximal convoluted tubules desquamation, cellular casts, and regenerating tubular epithelium were observed. The adrenal glands were normal in both of these animals. Dog no. 3 was continued on the low intake of yeast 2019 until the fiftieth

week when he was sacrificed. Sections of the liver, kidney and adrenals were normal in this animal.

During this study neither the dogs receiving the low yeast intake nor the inanition controls showed any increased retention of bromosulphalein or serum phosphatase, and sections of the liver were entirely normal.

Experiment 2

Normal protein diet (20% casein) with minimal yeast supplement

The 8 animals in this study received the 20% casein diet and, following a control period of 5 weeks, were divided into 3 groups as in experiment 1. The 3 vitamin B-complex deficient dogs received 0.1 gm of yeast 2019 per kilo of body weight per day. The 3 normal animals and the 2 inanition controls continued to receive 4 gm of type 3 yeast per day.

Dogs with subnormal yeast intake. All 3 animals (group 3) receiving yeast 2019 showed a decrease in food intake. Dog no. 9 developed a marked and rapid decrease in food intake, so that on the one hundredth day he vomited, showed signs of toxemia, and soon was unable to stand. The same day he was given an injection of a mixture of B vitamins with the following composition: thiamine, pyridoxine and riboflavin, 300 μg of each; pantothenic acid, 1.5 mg; nicotinic acid, 7.5 mg; inositol, 15 mg; and choline, 75 mg. The dog recovered completely the next day, but during succeeding weeks his food intake increased, and remained at a much higher level than that observed in the other 2 animals (figs. 2, 3 and 4).

During the first 27 weeks of the experiment dogs 10 and 11 showed definite increases in bromsulphalein retention, and dog 9 showed an increase in serum phosphatase (figs. 2, 3 and 4). Similar changes in the functional tests did not occur in the previous experiment in which the dogs received a 41% casein diet. At the beginning of the twenty-seventh week the daily supplement of yeast was reduced by one-half, and produced a further increase in dye retention and serum phosphatase

(figs. 2, 3 and 4). In dog no. 11 serum phosphatase did not increase significantly above normal. A liver biopsy taken during the thirty-second week showed large fat droplets generally distributed throughout the lobule, but with some tendency to central accumulation.

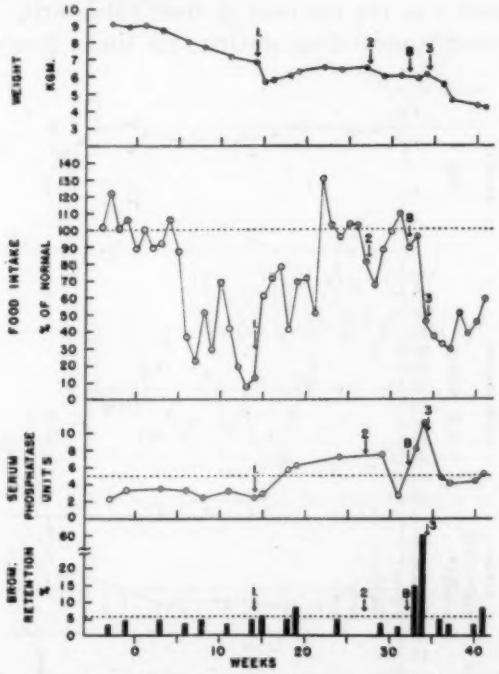

Fig. 2 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake with a normal (20%) casein diet. Data on dog no. 9, receiving 0.1 gm/kg/day of yeast 2019, started at zero time. At 1, an injection of B vitamins was given (see text). At 2, the yeast intake was reduced by one-half. B indicates liver biopsy. At 3, daily supplements of yeast extract were started and total caloric intake was restricted.

Dog 10 showed a rapid decline in food intake during the thirty-third week and died on the two hundred and thirty-first day of the study. For 2 days before death he was given injections of crystalline B vitamins but would not eat during

that time. At the end of the thirty-third week the other 2 deficient dogs were treated with 4 gm of type 3 yeast extract per day. During this period the food intake was limited to 22 cal. per square meter per hour, so that caloric or protein intake would not increase. This feature is represented in figures 2 and 3 as the per cent of food voluntarily consumed by the normal control dogs during this time. Dog no. 9 still

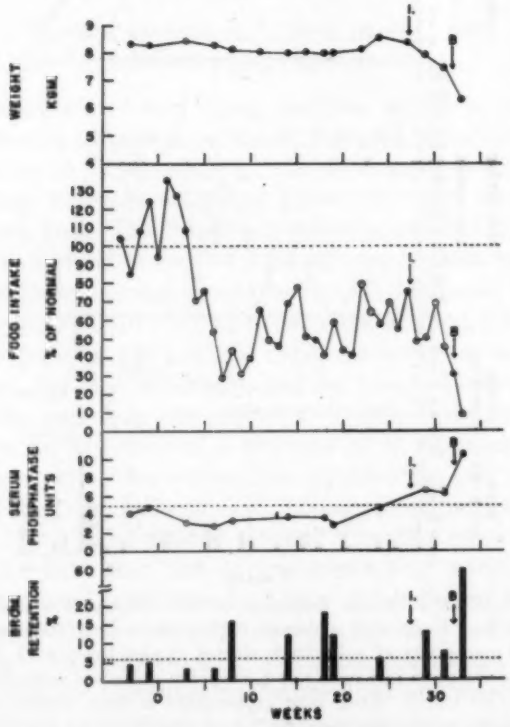

Fig. 3 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake, with a normal (20%) casein diet. Data on dog no. 10, receiving 0.1 gm/kg/day of yeast 2019, started at zero time. At 1, the yeast intake was reduced by one-half. B indicates liver biopsy. After the last functional test in the figure, therapy with B vitamins was started, but the dog died before further functional tests were made (see text).

lost weight on this food allowance so that during the thirty-seventh week he was given 30 cal. per square meter per hour. The dye retention and serum phosphatase of both dogs returned to normal. When these animals were sacrificed during

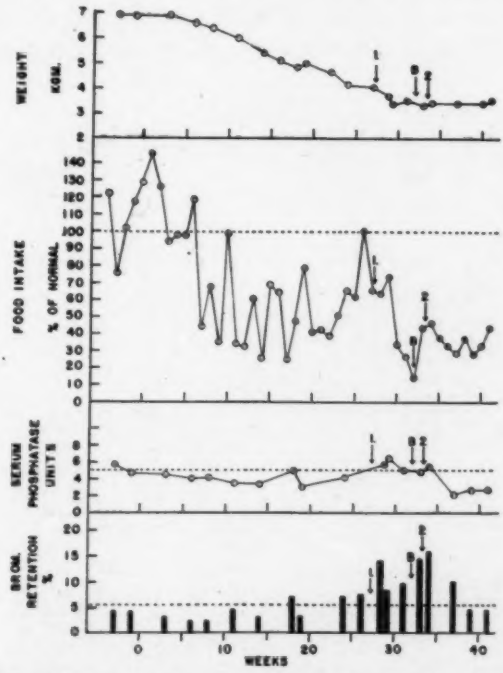

Fig. 4 Effect of a chronic deficiency of the vitamin B complex on liver function and food intake, with a normal (20%) casein diet. Data on dog no. 11, receiving 0.1 gm/kg/day of yeast 2019, started at zero time. At 1, the yeast intake was reduced by one-half, B indicates liver biopsy. At 2, daily supplements of yeast extract were started and the total caloric intake was restricted. Compare with figure 1.

the forty-second week the livers were found to be normal histologically (plate 1).

Inanition control dogs. These dogs (nos. 12 and 13) did not show any significant changes in liver function and their

hepatic histology remained normal. On 3 occasions during the study the bromsulphalein retention was slightly above normal, but the change was not consistent and was under 10% (fig. 5). On 5 occasions the serum phosphatase was over 5 units but not above 6 units. Liver biopsies during the thirty-second week and terminal studies of the liver during the forty-second week showed a normal histology. When the food intake of the

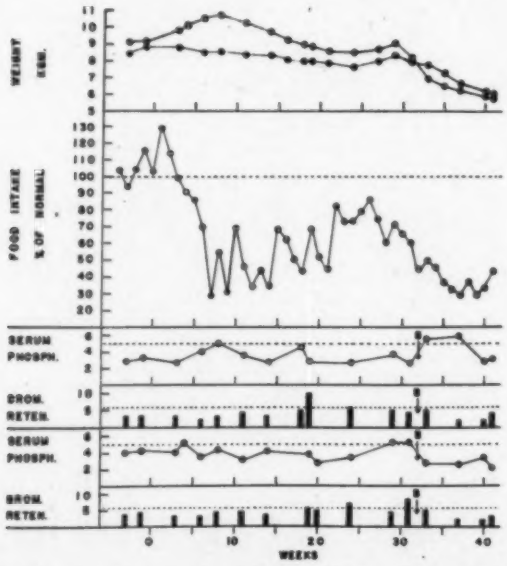

Fig. 5 Effect of inanition on liver function, with a normal (20%) casein diet, and with adequate supplements of yeast extract. B indicates liver biopsy (dogs nos. 12 and 13).

deficient dogs was restricted during the period of yeast therapy, the inanition controls were similarly limited to 22 cal. per square meter per hour.

Normal control dogs. The weekly average caloric intake for these 3 dogs (nos. 14, 15 and 16) is shown in figure 6. The bromsulphalein retention remained consistently below 6% in all 3 dogs with only 1 exception (fig. 6). On 5 occasions the

serum phosphatase was above 5 units, the highest value being 5.8 units. Liver biopsies during the thirty-second week and terminal studies of the liver during the forty-second week showed normal hepatic histology.

Kidney and adrenal glands. The 3 dogs on the low yeast intake developed renal changes consisting of proliferation of

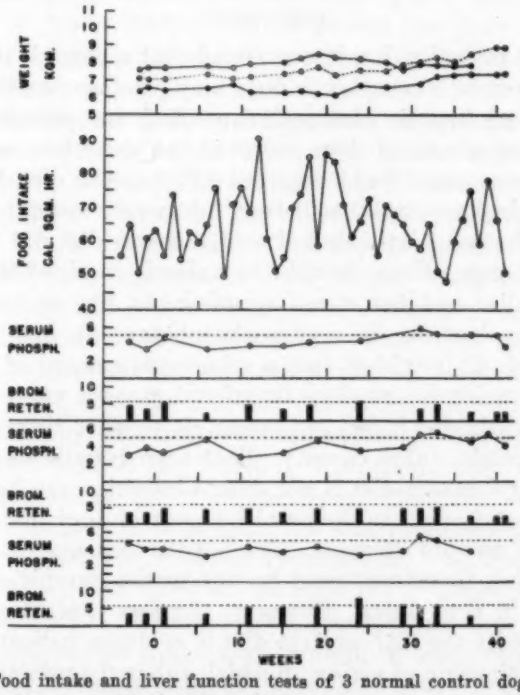

Fig. 6 Food intake and liver function tests of 3 normal control dogs (nos. 14, 15 and 16) with a normal (20%) casein diet.

the epithelial cells of the glomerular tuft and Bowman's capsule with crescent formation. Exudate was also present in the glomerular space. These changes were present in about 50% of the glomeruli and were most prominent in dog no. 9. This animal also had focal scars and periglomerular fibrosis, indicative of spontaneous healed pyelonephritis. None of the

above changes were observed in either the inanition-control dogs or in the normal control animals. There was no change in the adrenal tissue stained with hematoxylin-eosin, but in Sudan III preparations dog no. 10 showed depletion of fat in the fascicular and reticular layers. One of the inanition control dogs also had depletion of fat in the outer fascicular layer.

DISCUSSION

In this investigation it was found that a chronic deficiency of the vitamin B complex in dogs may produce functional and histological hepatic changes, depending on protein intake. When each group of dogs received the same low amount of yeast, it was noted that 3 dogs fed a 20% casein diet developed changes in liver function before the twenty-seventh week of study, whereas 3 animals fed a 41% casein diet did not show such a change. Even though the animals received the higher protein diet and low yeast supplements for as long as 50 weeks, no increase in serum phosphatase or dye retention developed. It is evident that a subnormal amount of the vitamin B complex can produce functional changes when a normal (20%) casein diet is fed, but cannot do so when there is a high protein intake (41% casein). That such hepatic dysfunction on the 20% casein diet is not due to inanition can be seen by the lack of changes in the inanition-control dogs that received a normal amount of yeast. The hepatic changes on the 20% casein diet, therefore, must be due to the chronic deficiency in vitamin B complex. However, the fact that they did not develop with the high protein diet is evidence indicating some interrelationship of action, in which either the vitamin B complex or the protein level of the diet is the deciding factor. This relationship may probably be explained by the fact that a low protein diet will reduce the stores of some of the B vitamins in the liver (Wright and Skeggs, '46; Sarett and Perlzweig, '43; Czaczkes and Guggenheim, '46).

When the yeast supplement of the deficient dogs fed the 20% casein was reduced, there was a further change in either the bromsulphalein or serum phosphatase tests. Liver biopsies

were then performed on all animals in this group, but only the yeast-deficient animals showed fatty changes. No hepatic necrosis was observed. The animals were then treated with yeast extract in the same amounts that the normal dogs and inanition controls were receiving. To prevent any increase in protein intake during this period both the vitamin-deficient group and the inanition-controls were given only limited amounts of food. Under such conditions the liver function tests returned to normal and the liver was free of its previous fatty change.

It is of interest that the dogs with functional hepatic changes showed only large fat droplets in the liver and no necrosis. This is in accord with the recent studies of Himsworth and Glynn ('44a, b). The observations of Gillman and Gillman ('45a, b) from South Africa are also pertinent. They studied the fatty changes in liver biopsy specimens from pellagrins and noted that therapy with riboflavin and nicotinamide increased the liver damage. They also mentioned that the hepatic picture became worse on treatment with brewers' yeast, nicotinic acid and riboflavin. No mention was made of therapy with brewers' yeast alone. When they tried liver extract rich in the fraction G of Cohn, they obtained a slow removal of fat from the liver. However, the most rapid disappearance of hepatic fat was produced by treatment with dried hog stomach (ventriculin). Methionine, in 2 cases, was without effect on the liver fat (Gillman, Gillman and Brenner, '45). These clinical reports also point to the fact that substances other than protein or methionine have a profound effect on at least 1 type of fatty liver. This finding, in general, is in agreement with our results on the relationship of protein intake and a chronic deficiency of the vitamin B complex with respect to functional and histological changes in the liver. This conclusion is not to be interpreted as denying the important role of protein and related substances in liver metabolism, but to point out under controlled conditions the as yet little known action of other substances.

SUMMARY

1. A *chronic* deficiency of vitamin B complex in dogs fed a high protein (41% casein) diet produced a decrease in voluntary food intake, but liver function tests and hepatic histology remained normal.

2. A similar *chronic* deficiency of vitamin B complex in dogs on a normal (20% casein) diet produced changes in serum phosphatase and bromsulphalein retention. These changes were not present in inanition-control dogs, fed a similar diet plus an adequate amount of vitamin B complex in the form of yeast. A further reduction of the yeast supplement of the chronically deficient animals produced further changes in the functional tests. Liver biopsies showed the presence of large fat droplets but no necrosis. Inanition-controls showed neither functional nor histological changes.

3. The chronically deficient animals were treated with yeast extract, which restored the liver function to normal and produced a disappearance of the large fat droplets in the liver, even though the protein and caloric intakes were restricted during the period of therapy.

4. It is evident that an interrelationship exists between protein intake and a chronic deficiency of the vitamin B complex. Indeed, under conditions of controlled inanition a chronic deficiency of the complex can be shown to play a definite role in producing liver injury.

ACKNOWLEDGMENTS

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PLATE 1

EXPLANATION OF FIGURES

Fatty change in the liver resulting from a chronic deficiency of the vitamin B complex. Tissues fixed in 10% formalin, stained with Sudan III, and photographed through a red filter. × 290.

1 Dog no. 12, inanition control, liver biopsy, thirty-second week.

2 Dog no. 15, normal control, liver biopsy, thirty-second week.

3 Dog no. 9, chronic deficiency of vitamin B complex, liver biopsy, thirty-second week.

4 Dog no. 9, forty-second week, after treatment with yeast extract.

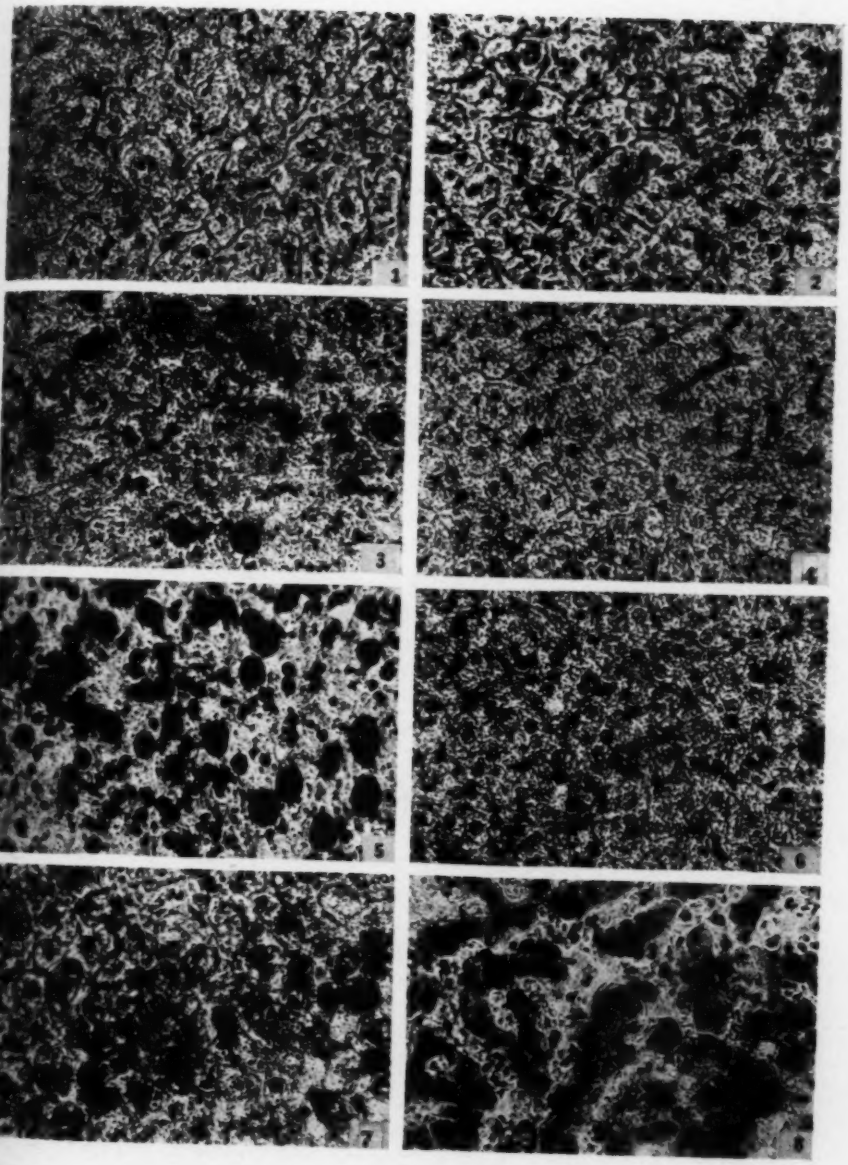
5 Dog no. 11, chronic deficiency of vitamin B complex, liver biopsy, thirty-second week.

6 Dog no. 11, forty-second week, after treatment with yeast extract.

7 Dog no. 10, chronic deficiency of vitamin B complex liver biopsy, thirty-second week.

8 Dog no. 10, forty-second week, died 2 days after treatment with B vitamins was started.

Figures 1 and 2 are representative of the other inanition control and normal dogs in the experiment.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PANTOTHENIC ACID IN THE NUTRITION OF THE HEN

M. B. GILLIS, G. F. HEUSER AND L. C. NORRIS
Agricultural Experiment Station and School of Nutrition, Cornell University, Ithaca, New York

TWO FIGURES

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Pantothenic acid has been shown to be necessary for reproduction in the hen. Gillis, Heuser and Norris ('42) found that when hens were fed a diet of ordinary feedstuffs heated to destroy pantothenic acid their eggs failed to hatch unless this factor was supplied. Studies have also been made of the quantitative pantothenic acid requirement of hens fed a heated diet (Gillis, Heuser and Norris, '43). During the course of these studies, however, it became evident that optimum hatchability could not be obtained with such a basal diet even when it was supplemented with adequate amounts of pantothenic acid. Under these conditions the requirement appeared to lie between 1200 and 1700 μg per 100 gm of diet. When these results were published, it was emphasized that the requirement for pantothenic acid may have been augmented by the deficiency of some other factor or factors in the heated diet. In the absence of other experimental data, however, these results have sometimes been used as a basis for making recommendations concerning the pantothenic acid content of breeder diets for hens.

In an attempt to formulate diets composed of natural feeds without heat treatment, ingredients were assayed and selected on the basis of a low pantothenic acid content. It was

not possible, however, to formulate a satisfactory diet containing less than 500 μg of pantothenic acid per 100 gm of diet. The results of limited trials with this type of diet suggested a requirement of between 800 and 1000 μg of pantothenic acid per 100 gm of diet for satisfactory hatchability (unpublished results).

In view of the need for more exact information on the requirements of the hen for pantothenic acid, further studies of this problem were undertaken. For this purpose the heated diet of natural feeding-stuffs was replaced by one consisting chiefly of purified ingredients supplemented with the necessary vitamins and minerals. The results reported in this paper include observations on the requirements for maintenance, egg production, and hatchability as well as the effects of different dietary levels of pantothenic acid on the amount of this factor in the hen's blood and eggs, and the effect on the viability of the progeny.

EXPERIMENTAL PROCEDURE

The work described in this report consists of 2 experiments conducted during consecutive years. White Leghorn hens in their first year of production were used as experimental animals in both studies. Before beginning each experiment the hatchability and egg-laying capacity of the hens were determined while they were maintained on a commercial breeder diet. The experimental hens were then distributed on the basis of hatchability, egg production, body weight, and general physical condition in order to insure the greatest possible uniformity between the lots in each experiment.

The hens were housed in wire-floored pens with feed and water supplied ad libitum. In the first experiment 6 lots of 8 hens and 1 male each were maintained on the experimental diets for 10 weeks. In the second experiment, which was carried on for a period of 15 weeks, 6 lots of 10 hens and 2 males each were used. In both experiments the males were rotated frequently among the different lots in order to elimi-

nate differences in breeding capacity. Individual records of production and hatchability were kept throughout each experiment. Eggs were incubated weekly and fertility and embryonic mortality were determined by candling on the seventh, fourteenth, and eighteenth days of incubation. At the time of the last candling the eggs were transferred to pedigree hatching trays.

During the second experiment, observations were made also on the effects of different dietary levels of pantothenic acid on the amount of this factor in the hen's blood and eggs. Eggs and blood from all lots were analyzed for pantothenic acid at intervals as described below. During this experiment the growth and livability of chicks produced by hens receiving different levels of pantothenic acid were also studied.

The basal diet used in these experiments had the following percentage composition: corn starch 59, crude casein 15, gelatin 5, fish meal 4, cellophane 4, soybean oil 3, cod liver oil 0.5, oyster shells 2, limestone 2, dicalcium phosphate 3, salt mixture 2 [1] and vitamin supplements 0.5.[2] Fish meal was included in the diet to furnish the factor in liver paste which was previously found to be necessary for hatchability (Gillis et al., '42), and which subsequent work by a number of investigators has indicated to be present in fish meal and other animal proteins. Microbiological assay of this diet, using *Lactobacillus arabinosus* as the test organism, showed it to contain approximately 1.5 μg of pantothenic acid per gram (pantothenic acid as used here refers to calcium pantothenate, dextrorotatory). The different levels of supplemental calcium pantothenate, in aqueous solution, were added to the basal mixture at weekly intervals to prevent destruction in storage.

[1] Salt mixture per 100 pounds: K_2HPO_4 420 gm, NaCl 275 gm, $MgSO_4 \cdot 7H_2O$ 185 gm, $FeSO_4 \cdot 7H_2O$ 19 gm, $MnSO_4 \cdot 4H_2O$ 5 gm, KI 1.5 gm, $ZnCl_2$ 1 gm, $CuSO_4 \cdot 5H_2O$ 1 gm, $CoCl_2 \cdot 6H_2O$ 0.5 gm.

[2] Vitamins per 100 pounds: thiamine 0.15 gm, riboflavin 0.22 gm, pyridoxine 0.22 gm, niacin 1 gm, folic acid 0.1 gm, vitamin K 20 mg, biotin 7 mg, inositol 40 gm, wheat germ oil 70 gm, choline 100 gm.

RESULTS

Hatchability, egg production and maintenance

The results on hatchability in both the first and second experiments are given in table 1. In the first experiment the requirement for optimum hatchability was between 750 and 1000 µg pantothenic acid per 100 gm of diet. However, in the second experiment, 650 µg resulted in as good hatchability as any of the higher levels. These results indicate a considerable genetic variation among hens in their pantothenic acid

TABLE 1

Average hatchability of fertile eggs from hens receiving different levels of pantothenic acid (µg pantothenic acid per 100 gm of diet).

Weeks inclusive	EXPERIMENT 1					
	150 µg	250 µg	500 µg	750 µg	1000 µg	1500 µg
	%	%	%	%	%	%
1st–3rd	93	80	90	93	92	82
4th–7th	38	57	72	79	87	80
8th–10th	0	11	52	56	85	79
	EXPERIMENT 2					
	150 µg	350 µg	650 µg	800 µg	950 µg	1100 µg
	%	%	%	%	%	%
1st–3rd	95	87	92	93	95	95
4th–7th	20	84	91	91	96	91
8th–10th	6[1]	75	91	91	94	92
11th–15th	..[2]	76	95	92	92	92

[1] 10th week, zero. [2] Discontinued.

requirement. This is not surprising in view of the wide variation which several investigators have found in the pantothenic acid requirements of young chicks. The lower requirement for the hens in the second experiment can be explained, in part at least, by the degree of selection to which they were subjected. The number of hens with preliminary hatching records available was considerably greater for the second experiment than for the first, resulting in a more rigid selection of the hens for good hatchability.

In both instances the hatchability of eggs from the basal lot receiving 150 µg of pantothenic acid per 100 gm of diet

declined to zero. The complete failure of hatchability on this deficient diet is further evidence of the vital role of pantothenic acid in reproduction. The excellent hatchability obtained when the basal diet was supplemented with adequate pantothenic acid indicates that the diet used is probably adequate in all nutritional factors required by the hen other than pantothenic acid. The results obtained in these experiments involving a purified diet show that the pantothenic acid requirement of hens is considerably lower than that obtained on the diet of heated natural feedstuffs. A deficiency in the heated diet of some other factor required for hatchability undoubtedly operated to increase the requirement for pantothenic acid.

A deficiency of pantothenic acid in the diet of hens did not result in characteristic deformities in chick embryos such as are obtained with a riboflavin deficiency. Although Taylor, Mitchell and Pollack ('41) have reported slightly abnormal or subnormal development in certain chick embryonic tissues and organs, gross inspection of embryos which failed to hatch did not reveal any characteristic syndrome. Poor quality of down, however, was sometimes observed in the embryos which failed to hatch as well as in some of the chicks hatched on the lower levels of pantothenic acid. In these studies it was again impossible to find any support for the claim of Taylor et al. ('41) that pantothenic acid favorably affects embryonic survival during the first 13 days of incubation. Practically all of the mortality in these experiments occurred after the candling on the eighteenth day. Many of the embryos that failed to hatch were alive on the twenty-first day and actually succeeded in pipping the egg shell. Thus, most of the embryos appear to be able to develop even when a pantothenic acid deficiency exists in the egg, but when they are required to break out of the shell, they are unable to perform this task.

The decline in hatchability of fertile eggs did not keep pace with the rate of depletion of the hens and of their eggs in pantothenic acid. This strongly suggests that at least part

of the effect of a pantothenic acid deficiency is due to a disturbance of the reproductive physiology aside from a lowering of the pantothenic acid content of the egg. A longer period is required to bring about this disturbance than is needed to deplete the hen of pantothenic acid which is not stored to any appreciable extent. The administration of pantothenic acid to deficient hens, however, resulted in an immediate resumption of hatchability. This is illustrated by the basal lot in the second experiment. This lot which received the unsupplemented diet declined in hatchability from 97% to zero, and was taken off the experiment after the tenth week. The hens from this lot were divided into 2 equal groups which were fed the basal diet supplemented with calcium pantothenate in

TABLE 2

Percentage hatchability following administration of pantothenic acid (P.A.) to depleted hens.

DEPLETION PERIOD		RECOVERY PERIOD			
P.A. per 100 gm diet	Hatchability (10th wk.)	P.A. per 100 gm diet	Hatchability (11th wk.)	(12th wk.)	(13th wk.)
μg	%	μg	%	%	%
150	0	800	40	96	93
150	0	1100	71	91	88

sufficient amount to raise the total to 800 and 1100 μg per 100 gm of diet, respectively, for the remaining 5 weeks of the experiment. Their immediate recovery of hatchability is shown in table 2.

The effect of different levels of pantothenic acid on the rate of egg production was observed in both experiments. In neither experiment was there a correlation between egg production and the levels of pantothenic acid fed. Although hens were not maintained on the basal diet longer than 10 weeks, no decrease in egg production was observed during this period. During the second experiment, egg production actually increased appreciably during the 10-week period that the hens were maintained on the basal diet. The requirement for egg production, therefore, is considerably less than for hatch-

ability and does not appear to be greater than 150 μg of pantothenic acid per 100 gm of diet. It is possible, however, that the requirement might be found to be greater if hens were maintained on such a low level of pantothenic acid for a more protracted period.

The average weight of the hens in all lots in both experiments increased during the experimental period and their general physical condition was good at the end of the feeding trial. The response of the basal lot was as good in this respect as that of any of the lots receiving pantothenic acid supplements, although this lot was not maintained beyond 10 weeks in either case. Mortality was negligible in all lots. The results of these experiments suggest that the maintenance requirement of the adult fowl is not greater than 150 μg of pantothenic acid per 100 gm of diet. This is about 25% of the requirement of the rapidly growing chick.

Effect of level of pantothenic acid in the hen's diet on the viability and growth of the chicks produced

Pantothenic acid deficiency does not seriously interfere with the development of the chick embryo until near the time of hatching. As a consequence of the ability of the embryo to develop even when the egg is deficient in pantothenic acid, many chicks actually hatch from eggs containing suboptimum levels of pantothenic acid. Most of the chicks from hens receiving the lower levels of pantothenic acid were weaker when hatched than were chicks from hens receiving more pantothenic acid. This has been a constant observation in experiments with both the heated and purified diets. An optimum allowance of any nutritive factor for breeding animals is one which permits the production of the greatest number of vigorous progeny. Therefore, the requirement of breeding hens for pantothenic acid is not simply for hatchability, but for the production of chicks strong enough to live and make rapid gains when properly fed. It is important that

the newly-hatched chick have sufficient body stores of pantothenic acid to last it until this factor can be assimilated in proper amounts from the food it consumes. Williams et al. ('41) have shown that the tissues of the chick are lower in pantothenic acid content at the time of hatching than during embryonic development or later life.

A study was made to determine the level of pantothenic acid in the hen's diet which results in the lowest mortality and most rapid growth of the progeny. During the eleventh and again during the thirteenth week of the second experiment 20 chicks were selected from each lot (except the basal

TABLE 3

Effect of pantothenic acid content of the hen's diet on survival and early growth of chicks.[1]

P.A. PER 100 GM HEN'S DIET	WEIGHTS OF CHICKS AT 4 WEEKS	MORTALITY
μg	gm	%
350	253	50
650	268	0
800	281	0
950	304	0
1100	296	0

[1] Average of 2 experiments — 40 chicks.

which had ceased to hatch) for use in these studies. As nearly as possible an equal number of chicks from each hen was used. The chicks from each lot were weighed, wing-banded, and placed in electrically heated battery brooders when 1 day old. All lots were given the same practical type diet containing approximately 1400 μg of pantothenic acid per 100 gm.

The results on growth and mortality of chicks from all lots are given in table 3. Mortality was encountered only in the lot from hens receiving 350 μg of pantothenic acid per 100 gm of diet. Both survival and growth response were very poor in this lot, leaving no doubt as to the inadequacy of this level of pantothenic acid for breeding hens. In addition to heavy mortality and retarded growth, feathering was very poor in

this lot at the end of the fourth week. About 50% of the chicks surviving in this group showed signs of a mild dermatosis at some time.

Growth of chicks from the lot receiving 650 μg of pantothenic acid was less satisfactory than that of chicks from lots receiving more of this vitamin. A few of the chicks from this lot exhibited some nervous disorders, such as turning the head from side to side or tremors; however, there were no symptoms of dermatosis. It appears that at least 800 μg of pantothenic acid in 100 gm of the hen's diet is necessary to produce the most viable chicks capable of making rapid early gains in weight. This conclusion is based on the fact that 800 μg prevented all symptoms of deficiency in the chicks and permitted growth which was not significantly below that of chicks from lots receiving more pantothenic acid. The growth of chicks from the lot receiving 650 μg was significantly below that of chicks from the 2 lots receiving the highest levels of pantothenic acid.

Influence of the diet on the pantothenic acid content of the hen's blood and eggs

The effect of the dietary level of pantothenic acid on the amount of this factor in the hen's blood and eggs, as well as on the rate of depletion was studied during the second experiment. Microbiological assays for pantothenic acid were conducted on both blood and eggs from all lots at the beginning of the experiments and at intervals thereafter. The assay procedure was a modification of the method of Skeggs and Wright ('44). Since preliminary tests showed that enzymatic treatment of these materials resulted in no increase in pantothenic acid content, this step was eliminated in the assays reported here.

One-milliliter samples of blood were withdrawn from the wing veins of each hen for assay purposes. The samples of whole blood from 3 or 4 hens were then combined, diluted with water, autoclaved, filtered, and suitable aliquots of the filtrate

used for the assays. The procedure with eggs was as follows: 3 or 4 eggs were broken into a graduated cylinder, weighed, and the volume adjusted with distilled water to 1 ml per gram of whole egg. The whole eggs were then homogenized in a Waring Blendor, and an aliquot of the homogenous material diluted with distilled water, autoclaved, and filtered. The filtrate was used for the assay.

The pantothenic acid content of the diet was quickly reflected in the amount of this factor found in the blood. At

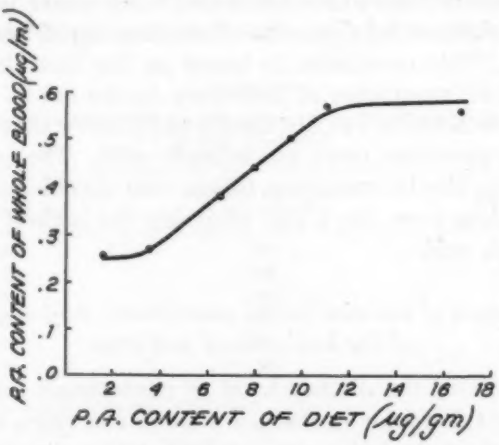

Fig. 1 The effect of different dietary levels of pantothenic acid on the amount of this factor in hen's blood.

the beginning of this experiment, the pantothenic acid content per milliliter of whole blood was approximately 0.56 µg for all lots. The amount of pantothenic acid in the blood became stationary within 3 weeks after the hens were placed on the experimental diet. The relationship between the pantothenic acid content of the diet and that of the blood is represented graphically in figure 1. The response of the blood to different levels of dietary pantothenic acid is a straight-line relationship within the limits of about 350–1100 µg per 100 gm of diet. Above or below these values, however, the curve flattens out. This sug-

gests that the animal exerts some control over the amount of this vitamin in the blood, probably through control of its excretion as certain limits are approached. Approximately 1100 μg of pantothenic acid were required in this experiment to maintain saturation of the blood with this factor. Since optimum reproductive performance was associated with a level of about 800 μg of pantothenic acid, saturation of the blood is not necessarily a criterion of adequate nutrition.

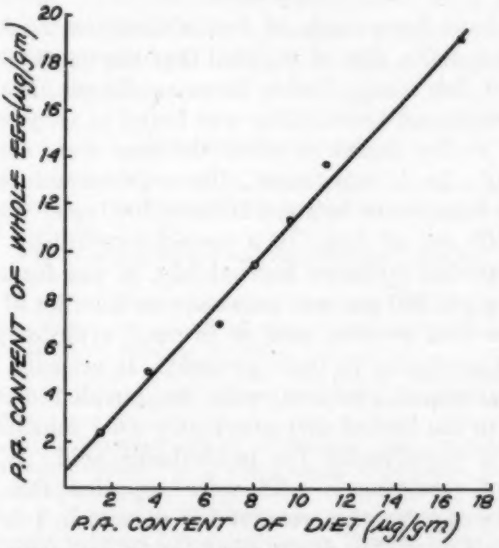

Fig. 2 The effect of different dietary levels of pantothenic acid on the amount of this factor in hen's eggs.

The pantothenic acid content of eggs is likewise immediately affected by the amount of this factor in the diet. The values for eggs did not decrease after the third week. The relationship between the dietary level of pantothenic acid and the amount of this factor in the egg is shown graphically in figure 2. It will be observed that a straight-line relationship exists between the pantothenic acid content of the diet and the eggs over the range of values studied in this experiment. This

relationship is expressed by the equation, $Y = 1.1\ X + 0.7$, in which Y equals the micrograms of pantothenic acid per gram of whole egg, and X equals the micrograms of pantothenic acid per gram of diet. These values agree fairly well with those reported by Pearson, Melass and Sherwood ('45). In this experiment, optimum results were associated with pantothenic acid levels of about 9.5 μg or more per gram of whole egg.

SUMMARY

Studies have been made of the pantothenic acid requirement of hens fed a diet of purified ingredients supplemented with 4% of fish meal. Under these conditions, the requirement for maximum hatchability was found to vary somewhat, depending on the degree to which the hens were selected for hatchability. In 1 experiment, the requirement for hatchability was found to lie between 750 and 1000 μg of pantothenic acid per 100 gm of diet. In a second experiment although 650 μg supported optimum hatchability, it was found that at least 800 μg per 100 gm was necessary in the diet of the hens to promote best growth, and to prevent symptoms of deficiency and mortality in their progeny. It appears from the lower requirement obtained with the purified diet, that a deficiency in the heated diet previously used considerably increased the requirement for pantothenic acid. When hens depleted of pantothenic acid were supplied this vitamin, hatchability of their eggs returned to normal in 1 or 2 weeks.

Embryonic mortality due to pantothenic acid deficiency was found to be confined almost entirely to the last 2 or 3 days of the incubation period. The deficiency did not result in deformities in the embryos. Chicks hatched from hens receiving suboptimal levels of pantothenic acid were generally inferior, however.

Egg production and maintenance of weight were not adversely affected during a 10-week period by a pantothenic acid level as low as 150 μg per 100 gm of diet. Hens maintained for 10 weeks on a diet containing this amount of pantothenic acid actually increased their body weight during 2

different experiments, and in 1 instance increased the rate of egg production.

The pantothenic acid content of the diet is quickly reflected in the amount of this factor found in the blood and eggs. The amount stored in eggs was found to fit the same straight-line equation for all dietary levels studied. The amount of pantothenic acid in the blood is much lower than that for eggs, and tends to remain constant below 350 μg per 100 gm of diet, or above 1100 μg. The minimum pantothenic acid content of whole blood associated with optimum reproductive performance was 0.45 μg per milliliter. For eggs, the corresponding value was 9.5 μg per gram of whole egg.

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AN ADJUSTABLE CYLINDRICAL CAGE FOR USE IN METABOLISM STUDIES WITH YOUNG PIGS

J. MILTON BELL[1]
Laboratory of Animal Nutrition, Cornell University, Ithaca, New York

TWO FIGURES

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The problem of adequate caging frequently arises when young pigs are to be employed in metabolism studies, and especially where accurate separation of excreta is an essential. For pigs over 50 pounds live weight the rectangular-type metabolism cage as used by Watson et al. ('43) and by Crampton and Whiting ('43) is very satisfactory and offers few collection difficulties. Pigs from weaning age to 50 or 60 pounds live weight probably resent confinement more than do heavier animals and, being considerably more agile, such young pigs have to be confined very closely when in rectangular cages in order to prevent them from turning.

With these facts in mind it seemed worthwhile to design a cage which would allow maximum freedom of movement and still permit separate collection of excreta from male pigs below about 50 pounds live weight.

DESIGN

General

The cage consists essentially of a rectangular strip of wire mesh bent into a cylindrical shape to form the walls, and anchored on a heavier wire mesh floor by means of expansion-

[1] The author wishes to acknowledge the assistance of M. J. Brinegar.

type coil springs. In order to confine the animal from above, and yet permit easy adjustment, the top is suspended from the ceiling by light chains at the back and with a chain and hook at the front for use when the top is up. Expansion coil springs also serve to retain the top in position when the cage is closed.

As support for the basic structure described above, a bench large enough to hold 4 cages was constructed. Underneath each cage a central funnel directs the urine to a collection bottle below the bench.

In this type of metabolism cage, feeding is accomplished by placing a suitable trough in the cage until the animal has finished eating.

Details of construction

Walls. One piece of $2' \times 9'$ flattened expanded metal [2a] is bent in the form of a cylinder of about 3′ diameter, and provided with 2 hooks or clasps for holding the cylinder at any desired size.

Top. This consists of a $3' \times 3'$ wooden frame of $2'' \times 2''$ supporting $\frac{5}{8}''$ iron rods 3″ apart. Two pieces of chain suspend the back of the top from the ceiling. Two expansion-type coil springs serve to hold the top in position when the cage is closed.

Floor. Four pieces of $3' \times 4'$ flattened expanded metal [2b] are laid on a $3' \times 16'$ frame of $2'' \times 4''$ lumber, with $\frac{3}{16}'' \times 1\frac{1}{2}''$ strap iron cross-braces at 12″ intervals throughout the length of the frame. This frame is provided with angle-iron legs to hold the cage floors 9″ above the bench. This permits easy collection of the feces which fall through the floor.

Bench. This is 28″ high × 40″ wide × 16′ long, well-braced and provided with $2'' \times 4''$ legs at intervals of 4′.

[2] Wheeling Corrugating Company, Buffalo, N. Y.
a. Design no. 9–11, $1\frac{1}{2}''$ flattened expanded metal (plain).
b. Design no. 9–11, $\frac{3}{4}''$ flattened expanded metal (plain).

On the bench under each cage, a galvanized metal sheet serves to provide an easily cleaned surface for the fecal collections. A gradual slope to the sheet, and facilities for collecting liquid excreta have proven useful, especially while the cage is being adjusted to the animal for the first few days.

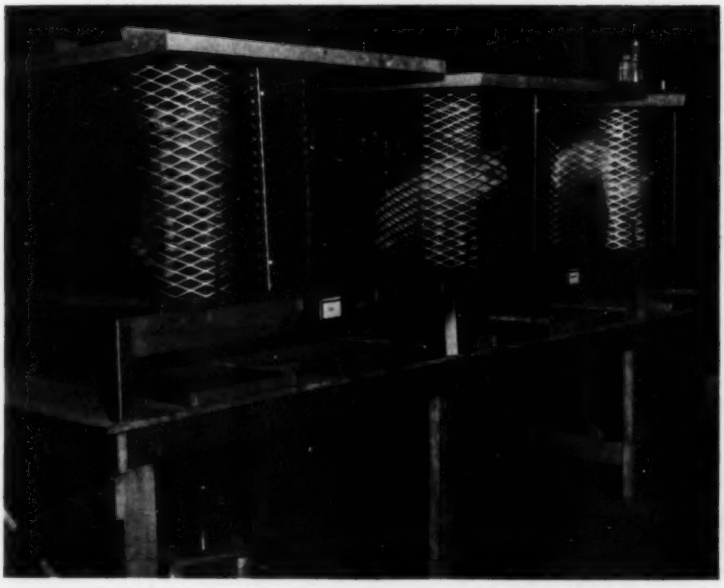

Fig. 1 Front view of a battery of cages in use.

Funnel. It is 16″ in diameter, placed directly under the center of the cage below the floor. It is shallow and has a short stem to permit easy removal. A rubber connection below the bench directs the urine into the bottle. Each funnel possesses a screen and, at the bottom, some coarse glass wool to insure further against contamination.

Feeder. The sides are 16″ high sloping to 4″ high at the front, with a base of 7″. Two such sides together with a 9″ × 16″ back and a 4″ × 9″ front piece enclose a metal,

water-tight, round bottom feed container. A small hook at the back and 2 screws protruding ½″ down from the bottom adequately anchor the feeder while it is in the cage.

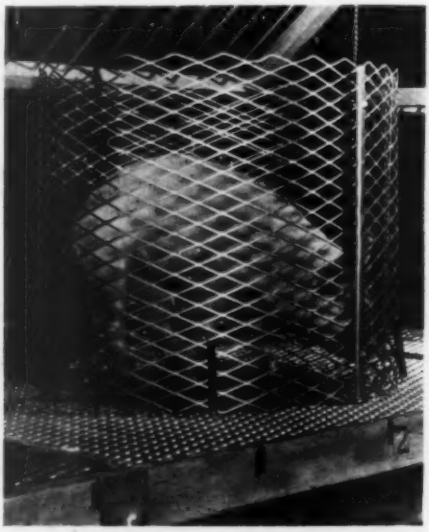

Fig. 2 Close-up view of a pig in a cylindrical cage. The top is up, ready for feeding or handling. The method of controlling the circumference of the cage is shown in the front.

DISCUSSION

While these cages have been in use pigs ranging in weight from 20 to 90 pounds have been observed. The cages were ordinarily adjusted to the length of the pig during the preliminary period on a given diet, following which the central funnel system in all cases permitted a highly satisfactory separation of urine and feces. Most well-formed feces pass through the floor, and those which remain are easily removed by raising the wall of the cage.

Since the feeding and collection methods permit no contamination of feces with feed, a high degree of precision can

be expected in balance work. The control of coprophagy has offered no problem. It is most probable that pigs under these conditions will not consume feces except incidentally when eating spilled feed amidst excreta. The feeder described above tends to crowd the pig and does not encourage spilling of feed.

The experience of this laboratory with the adjustable cylindrical cage indicates that it is to be preferred to the rectangular-type cage for use with small male pigs employed in controlled digestion and metabolism studies.

SUMMARY

An adjustable cylindrical cage is described for use in metabolism studies with male pigs, particularly those below 50–60 pounds live weight. In the experience of the author, several months' trial has indicated that the central funnel arrangement for urine collection is a practical one, highly efficient in permitting separation and collection of excreta. It allows some freedom of movement. The usual preliminary period allows sufficient time for adjusting the size of the cage to the animal.

Details of construction and illustrations of the cages in use are included.

ACKNOWLEDGMENT

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DENTAL CARIES IN THE COTTON RAT

X. THE EFFECT OF FLUIDITY OF THE RATION [1]

E. POTTS ANDERSON, J. KNOX SMITH, C. A. ELVEHJEM
AND PAUL H. PHILLIPS

Department of Biochemistry, College of Agriculture,
University of Wisconsin, Madison

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In the course of investigations on dental caries in the cotton rat Schweigert and co-workers ('46b) found that this animal was free of carious lesions when fed a diet of liquid whole milk. This was in contrast to the extensive dental decay developed on dry rations high in sucrose or other fermentable carbohydrates. Milk was found to exert a protective action even in the presence of 5 or 10% of fermentable sugar, although caries scores on such rations were not zero as they were on a diet of liquid milk alone (Anderson et al., '47). The higher caries scores when a dry ration was fed seemed to indicate that fluidity of the ration might play an important part in this protection. Studies on various dry and liquid rations were therefore undertaken.

EXPERIMENTAL

The experimental groups were set up according to the procedure of Shaw and his associates ('44a), using weanling cotton rats (20–25 gm) from our stock colony. Litters were divided among the groups to allow for differences in caries susceptibility (Schweigert et al., '45). The animals were killed

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at the end of the 14-week experimental period and the incidence and extent of carious lesions determined by the method of Shaw and co-workers ('44a, '44b).

Control animals were fed a dry ration known to be highly cariogenic.[2] Dry milk rations were fed as dry whole milk and as dry skim milk plus butter. The latter was prepared by adding butter to non-fat dry milk solids to make a ration of 21.3% butterfat. Both of these rations were supplemented with 4% of 1:20 liver extract, and minerals were added to make the intake of these the same as that of the animals which had been fed liquid milk.[3] Since the minerals were added in solution, these rations were dried after the addition of minerals and butter and subsequently ground in a Wiley mill. This was done merely to produce a ration of even consistency, and no attempt was made to control particle size.

Two other groups of animals received the dried milk rations reconstituted with water to produce equivalent liquid milk rations. To make the composition approximate that of liquid whole milk the rations were made up to 12.5% solids (14.3 gm per 100 ml of water). The respective dry milk rations were each mixed with warm water in a Waring blendor and then put through a hand homogenizer. Since the greater bulk of the liquid rations reduced the intake of solids, a further addition of minerals was made to these rations to equalize the consumption of these dietary factors.

A further experimental group was fed the cariogenic control ration 802 suspended in water. The casein was ground to a finer consistency in a ball mill before being mixed with the fat and soluble constituents. This ration was then mixed with water in the same way as the reconstituted dry milk diets above, i.e., on a 12.5% solids basis. Liver extract was added at the usual level of 4% of the solids. This ration was also

[2] This ration (802) had the following percentage composition: sucrose, 67; casein, 24; corn oil, 5; and salts IV, 4. Four per cent of 1:20 liver extract was added at the expense of the entire ration. Adequate quantities of the B vitamins (McIntire et al., '44) were added to the ration, and each rat received 1 drop of halibut liver oil per week.

[3] A daily intake of 1 mg of iron and 0.1 mg of copper and of manganese.

put through the hand homogenizer. The treatment did not produce a true liquid but served to put the more insoluble solids into a fine suspension. All rations were fed *ad libitum*.

RESULTS

The data on growth rate and caries indices are given in table 1. Experimental animals are paired with their littermate controls in each case. The animals from each litter were divided as equally as possible between the control and experimental groups, and if there was an extra animal in either group the average of the other group was weighted accordingly.

Growth rates are given as average weekly gains for the first 6 weeks and for the total 14 weeks on experiment. All

TABLE 1

The effect of various dry and liquid rations on growth rate and on incidence [1] and extent [2] of carious lesions in the cotton rat.

RATION	NUMBER OF ANIMALS	AVERAGE WEEKLY GAIN		AVERAGE INCIDENCE	AVERAGE EXTENT
		6 wks.	14 wks.		
		gm	*gm*		
802 Dry cariogenic control	12	11	7	25	59 +
Dry whole milk mixture	12	11	6	8	13 +
802 Dry cariogenic control	14	10	7	24	57 +
Dry reconstituted whole milk	14	8	5	1	1 +
802 Dry cariogenic control	15	11	8	28	70 +
Dry skim milk plus butter	15	8	7	14	30 +
802 Dry cariogenic control	11	11	8	32	57 +
Dry reconstituted skim milk plus butter	11	7	5	1	1 +
802 Dry cariogenic control	10	11	7	24	60 +
802 Liquid cariogenic control	10	7	5	9	14 +

[1] The incidence is an expression of the number of fissures with carious lesions. Since the cotton rat has 40 fissures per head the maximum caries incidence therefore would be 40.

[2] The extent of the caries of each fissure is assigned values of 1 + to 5 +; 1 + to 2 + = lesions involving only the enamel; 3 + = lesions involving the enamel and dentine; 4 + to 5 + = lesions resulting in fractures. The extent of carious lesions as used herein represents the sum of the individual fissure values.

growth averages were computed as growth of males by raising the figures for females by 20% at 6 weeks and 15% at 14 weeks (Anderson et al., '47). All animals grew well, although growth was usually somewhat better on the control ration than on the experimental diets.

The data on caries indices show that scores for all animals on the experimental diets were below those for animals fed the cariogenic control ration 802. The relative effect of the various experimental diets can be determined by comparing the differences between control and experimental averages in each case.

The caries scores of the rats fed dry milk rations were higher than the zero scores previously found on liquid whole milk. The average caries scores on dry whole milk were somewhat below those observed on dry skim milk plus butter. Thus, animals on dry whole milk had an average incidence of 8 and an average extent of 13 + as against the animals on control ration 802 which had an average incidence of 25 and 59 +, while animals on dry skim milk plus butter showed an average score of 14 and 30 + as against the controls on diet 802 with an average caries index of 28 and 70 +. It can also be seen that when these dry milk rations were reconstituted and fed in liquid form scores were reduced to nearly zero in both cases. Likewise, when the control ration 802 was mixed with water and fed in a liquid form, caries scores were found to be less than those of animals fed the same ration in a dry state, the averages being 9 and 14 + for experimental animals and 24 and 60 + for the controls on diet 802.

DISCUSSION

It appears from these data that fluidity of the ration is a very important factor in dental decay in the cotton rat. In comparison with the zero caries scores found with liquid whole milk, the caries scores on the dry milk rations fed in these experiments are high, but the incidence and extent of lesions were again reduced when these rations were reconstituted and fed in liquid form. Similarly, the caries scores of

animals receiving the ration 802 in a liquid form are considerably lower than those of their controls fed the same ration in a dry state.

Earlier experiments in this laboratory, using a synthetic dry ration approximating milk solids in composition, indicated that this ration produced a low incidence and extent of caries, although the caries scores were higher than the zero scores found on liquid whole milk (Schweigert et al., '46b). The caries indices found here on dry whole milk are above those observed on the previous dry milk solids ration, and the average for the animals fed dry skim milk plus butter is still higher. It is difficult as yet to explain these differences. It is possible that dissimilarities in composition existed between the dry milks used here and the synthetic milk solids ration fed in earlier experiments. Also, the percentage of fat in the skim milk plus butter ration is slightly below that usually found in milk solids. These differences may account for some changes in caries scores, since the levels of carbohydrate, fat, and protein in the diet have been found to affect caries production (Schweigert et al., '46a, '46b).

Differences in physical state other than fluidity may also have been influential. The drying process may alter certain milk constituents and thus make the effect of dried milk different from that of synthetic milk solids. Similarly, the condition of the fat may have been important in the difference between dry whole milk and dry skim milk plus butter. Free fat or oil in the diet has been postulated by Rosebury and Karshan ('39) and by McCollum et al. ('39) to reduce dental caries by forming a mechanical coating over the tooth or food particle, thus protecting against bacterial action. It is possible that butter added to the dry ration and mixed by hand may not coat the food particle as effectively as butterfat dried as part of the whole milk.

Fat may also enter into the effect of fluidity, although the condition of the fat in the reconstituted rations is unknown. Probably more important, however, is the possibility that these fluid rations, by remaining in the mouth for a compara-

tively short time, afford a less favorable medium for fermentation by oral bacteria. The differences in growth rate indicate that a lower caloric intake may also have had some effect.

The averages on the reconstituted milk rations are not zero as they have been found to be on liquid whole milk, but the differences may be within the limit of error of our procedure. The method of reconstitution was not entirely satisfactory and such small differences might also be accounted for by variations in physical state between these rations and liquid whole milk.

This, of course, does not preclude the possibility that other factors may also play a role in the protective action of milk. It is probable that the higher levels of fat and protein may exert some protection, and the presence of lactose as carbohydrate may enter into the picture, since it appears that this sugar is somewhat less cariogenic than the more fermentable ones. In addition, the effects of minerals and vitamins have been only partially investigated.

SUMMARY

Data are presented on the caries indices of cotton rats fed various dry and liquid rations.

Animals fed dry whole milk and dry skim milk plus butter were found to have an incidence and extent of carious lesions higher than the zero scores previously observed in animals fed only liquid whole milk. Caries scores were again reduced to nearly zero, however, when these rations were reconstituted with water and fed in liquid form.

In these experiments the caries indices of animals on all the diets were below those of control animals receiving the dry ration known to be highly cariogenic. Scores were also lower than those of controls when animals were fed the control ration in a liquid form. These data indicate that fluidity of the ration is an important factor in producing dental caries in the cotton rat.

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THE NUTRITIVE QUALITY AND THE TRYPSIN INHIBITOR CONTENT OF SOYBEAN FLOUR HEATED AT VARIOUS TEMPERATURES [1]

R. J. WESTFALL [2] AND S. M. HAUGE

Department of Biochemistry, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania, and Department of Agricultural Chemistry, Purdue University, Lafayette, Indiana

TWO FIGURES

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In their fundamental studies of the nutritive value of proteins, Osborne and Mendel ('12) found that glycinin, a protein isolated from soybeans, was of good nutritive value for rats, in comparison with a number of other proteins. The isolated protein was prepared by the method of Osborne and Campbell (1898), without the use of heat at any stage. Later, adopting the same methods that they had followed previously, and using soybeans rather than the isolated protein, Osborne and Mendel ('17) found that the protein of raw soybeans was of poor quality, and that heating the soybeans in the presence of water improved their nutritive value considerably. The earlier of these observations seems to have escaped general attention.

Mitchell and Smuts ('32), Shrewsbury and Bratzler ('33), and others found that the protein quality of raw soybeans

[1] Journal paper no. 321 of the Purdue University Agricultural Experiment Station.

[2] Formerly Research Assistant, Department of Agricultural Chemistry, Purdue University. Now with Sharp and Dohme, Inc.

was improved by supplementation with cystine, and Hayward and Hafner ('41) found that methionine was even more effective in this respect. Johnson, Parsons and Steenbock ('39) showed that, although the fractions of N and of S absorbed by rats from heated soybeans were only slightly larger than the fraction of each element absorbed from raw soybeans, the retention of each element was greater with the heated soybean meal. They suggested that there is present in raw soybeans "a N and S containing complex which is absorbable but not usable for tissue building purposes." The work of Evans and McGinnis ('46) and of Evans, McGinnis and St. John ('47), however, indicated that chicks fed raw soybean meal excreted a large proportion of the absorbed S in the feces rather than in the urine. They found that the loss of cystine in the feces was especially large.

The presence of a substance in soybeans that inhibited the proteolytic action of pancreatin was demonstrated by Ham and Sandstedt ('44) and by Bowman ('44). Adding a concentrate of the inhibitor to an otherwise adequate diet decreased the growth rate of chicks, as shown by Ham, Sandstedt and Mussehl ('45). Klose, Hill and Fevold ('46) showed that a similar concentrate would decrease the growth rate of rats, although this concentrate was not specifically identified as an inhibitor of a proteolytic enzyme. A crystalline protein with strong trypsin-inhibiting activity was isolated from soybeans by Kunitz ('45, '46), who later ('47) characterized its properties, showing its strong affinity for trypsin and its much weaker affinity for chymotrypsin. Bowman ('46) presented evidence that more than 1 trypsin inhibitor is present in soybeans.

It appeared probable that the trypsin inhibitor caused the difference in nutritive value between raw and heated soybeans. The results of the present investigation confirmed this assumption. Studies were also made of the period and the temperature of heating necessary for the experimental preparation of soybean flour of optimal protein quality.

EXPERIMENTAL

A commercial solvent-extracted soybean flour [3] was used in this study. For heating the flour at temperatures below 100°C., a suspension of the flour in water was maintained thermostatically at the desired temperature. After the required period, the suspension was cooled rapidly, lyophilized, and the dry product ground to pass a 40-mesh screen. For heating at temperatures above 100°C. the autoclave was used, with the flour spread in a thin layer. After the heating period, the flour was air-dried and ground to pass a 40-mesh screen.

Soybean protein was prepared from the raw flour by the method of Smith and Circle ('39), using pH 6.7 for extraction, and pH 4.1 for precipitation, of the protein. A concentrate of the trypsin inhibitor was prepared by lyophilizing the supernatant solution remaining after the precipitation of the protein.

The nutritive value of the proteins was determined by the mouse method of Bosshardt, Ydse, Ayres and Barnes ('46). A well-heated soybean flour was used in preliminary experiments to determine the optimal dietary level for soybean protein. Since maximal utilization of N for growth was indicated at 12% protein (N × 6.25), this level was used for all diets in which the protein was derived mainly from soybeans. Diets containing casein provided 10% protein (N × 6.25). The results were calculated as grams of weight gained per gram of protein consumed. Analyses of the carcasses for N showed that the weight gains were not due to an excessive deposition of non-protein constituents in the tissues.

The percentage composition of the diets was as follows: cerelose 20, hydrogenated vegetable oil 25, salt mixture (Hubbell, Mendel and Wakeman, '37) 4, Cellu flour [4] 2, corn oil 2, liver extract [5] 1, choline chloride 0.2, a vitamin A, D, and E concentrate 0.1, a vitamin B mixture 0.0372, and 2-methyl-1,4-

[3] Nutrisoy XXX flour, generously provided by Dr. J. W. Hayward of the Archer-Daniels-Midland Company.

[4] Obtained from the Chicago Dietetic Supply House, Chicago, Illinois.

[5] Wilson's 1:20 liver extract.

naphthoquinone diacetate 0.001, together with the required amount of the protein source and enough white dextrin to make 100. The vitamin A, D, and E concentrate contained 41 gm of corn oil, 2 gm of α-tocopherol, and 7 gm of a fish-liver oil concentrate containing 65,000 U.S.P. units of vitamin A and 13,000 U.S.P. units of vitamin D per gram. The vitamin B mixture was composed of 200 mg of thiamine hydrochloride, 400 mg of riboflavin, 200 mg of pyridoxine hydrochloride, 1,000 mg of niacinamide, 1,100 mg of calcium pantothenate, 5,400 mg of inositol, and 1,000 mg of p-aminobenzoic acid.

Determination of trypsin inhibitor potency

A method was developed for determining the trypsin inhibitor potency of raw or heated soybean flours. The method was the same in principle as Anson's ('38) method for trypsin determination. It differed from the method recently described by Borchers, Ackerson and Sandstedt ('47) for the determination of soybean trypsin inhibitor in the use of casein as the substrate instead of hemoglobin. Crude trypsin,[6] referred to hereafter as "pancreatin" to avoid confusion with pure trypsin, was used instead of the pure enzyme, because of the difficulty of obtaining the latter. In this method, 5 ml of 2.5% casein [7] solution and 1 ml of an inhibitor solution, or of water, were placed in each of a series of tubes. After warming the tubes to 35°C., 1 ml of a standard solution of pancreatin was added to each tube, and the incubation continued for exactly 20 minutes. To each tube 10 ml of 10% trichloracetic acid was added, after which the tube was shaken and allowed to stand for 1 to 3 hours. The suspension was filtered through a dry paper, and a 10 ml aliquot taken for N determination by a micro-Kjeldahl method.

The pancreatin solution was standardized to release from 1.8 to 2 mg of N in the aliquot analyzed, in the absence of any

[6] Wilson's 1:300 trypsin.
[7] "Labeo" casein.

inhibitor. The N in the aliquot was proportional to the amount of pancreatin when less than 2 mg of the enzyme was used. To obtain reproducible results, it was necessary to use a standardized enzyme solution, which was preserved by freezing in small portions. Each portion was thawed just before using.

Provided the amount of inhibitor present did not reduce the amount of N released by more than 35%, the decrease of the N released was proportional to the amount of inhibitor used. The amount of N released in each tube containing the inhibitor was deducted from the value obtained in the absence of any inhibitor. The difference was divided by the amount of inhibitor material added. An average of 8 or more determinations was used to obtain the inhibitor potency, expressed as milligram of N per milligram of test substance.

In assaying the heated soybean flours, thorough extraction of the inhibitor was necessary to obtain reproducible results. Satisfactory extraction was obtained by adjusting a 4% to 5% suspension of the flour to pH 10.5 for 30 minutes. Subsequent dilution to give the desired concentration of the inhibitor lowered the pH sufficiently so that no neutralization was necessary. These conditions caused no destruction of the inhibitor.

The relationship between inhibitor potency and protein efficiency

Separate portions of soybean flour were heated for 30 minutes at 60°, 65°, 75°, 85°, 95°, and 108°C. to yield products of decreasing inhibitor contents. The protein efficiency and the trypsin inhibitor potency of each heated portion were determined. The results (fig. 1) indicate that within the limits of accuracy of the methods used, the nutritive value of the protein increased in proportion to the destruction of the inhibitor. The results with soybean flour of higher inhibitor potency than the flour heated at 60°C. are not reported, because the

mice lost weight to a variable extent with such levels of inhibitor in their diets.

The isolation of soybean protein by the method described leaves a considerable proportion of the inhibitor in the product. The protein efficiency of this preparation was in approximate agreement with the value expected from the results with partially heated flours (fig. 1).

The inhibitor concentrate was added to a diet with autoclaved soybean flour, at a level permitting comparison with the previous results. The agreement with the expected value

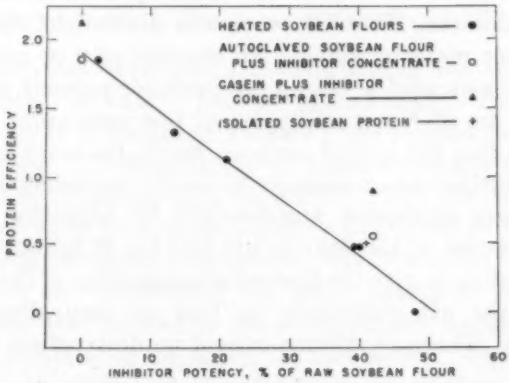

Fig. 1 The relationship between protein efficiency and trypsin inhibitor potency.

was also satisfactory (fig. 1). In this case, the inhibitor concentrate may have supplemented the autoclaved soybean flour to some extent, since the autoclaved concentrate, when fed with the autoclaved flour, gave a protein efficiency of 1.85 as compared with 1.65 for the autoclaved flour alone.

The effect of the inhibitor concentrate upon the nutritive value of casein was considerable, although proportionately less than upon that of soybean flour. In this instance, the inhibitor concentrate did not supplement the casein. These results also are given in figure 1.

The effect of different periods and temperatures of heating upon the nutritive value of soybean flour

Soybean flour was autoclaved at 108°C. (5 lbs.) and at 120°C. (15 lbs.) for different periods. The protein efficiencies of the products are shown by figure 2. It can be seen that, not only was the protein damaged by continued heating at 120°C. in the autoclave, but also that a soybean flour of optimal protein quality was not produced with any heating period at 120°C.

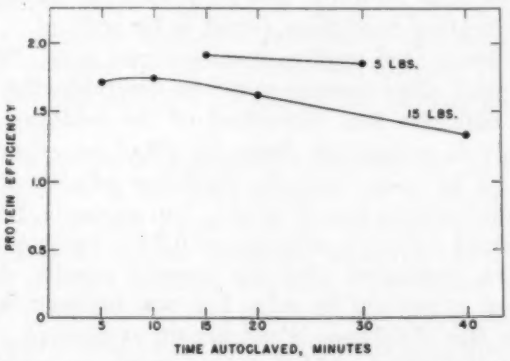

Fig. 2 The effect of different periods and temperatures of autoclaving upon the protein efficiency of soybean flour.

However, no significant loss of nutritive value resulted from extending the heating period from 15 minutes to 30 minutes at 108°C. Moreover, both results were essentially equivalent to the highest possible protein efficiency, as indicated by the extrapolation of the results for flours heated at lower temperatures.

DISCUSSION

Several of the observed relationships indicate that the trypsin inhibitor is the major cause of the poor utilization of the protein of raw or of partly heated soybeans. These relationships may be summarized as follows: (1) The protein efficiency of partially heated soybean flours increased in direct

proportion to the destruction of inhibitor potency, provided the temperature used was not over 108°C. (2) Adding the trypsin inhibitor concentrate to autoclaved soybean flour depressed the protein efficiency to the extent expected from the results with the partially heated soybean flours. (3) Isolating soybean protein by a process not involving heat improved the nutritive value to the extent expected from the proportion of inhibitor potency removed. (4) The highest protein efficiency attainable for the soybean flour used, as indicated by extrapolation to zero inhibitor potency, was the value reached under the heating conditions found to be optimal.

The evidence cited applies specifically to mice. While confirmation with other species would be desirable, the evidence available indicates that the effect of the inhibitor on mice differs only in magnitude from its effect on other species. However, it is clear that the inhibitor affects mice more strongly than it does rats or chicks. For example, Klose et al. ('46) obtained a protein efficiency of 0.7 for raw soybean flour fed to rats, compared with the present results showing a marked loss of weight by mice fed raw soybean flour. The reason for this difference is not known at present.

The harmful effect of high temperatures on soybean flour also may be greater for mice than for other species. Nevertheless, since the trypsin inhibitor is the cause of the poor utilization of raw soybean protein, the minimal heating conditions for destroying the inhibitor should yield a soybean flour of optimal quality for any species. Therefore, the determination of the trypsin inhibitor potency might be of practical value in controlling the heating processes used in the commercial production of soybean oil meal. Since the presence of a small amount of inhibitor potency would not have any significant effect, the optimal heating period for any particular process would be the time required to destroy all but a few per cent of the original inhibitor potency. However, the inhibitor determination cannot give any information regarding the deleterious effect of excessive heat. To measure this, other tests must be used.

The optimal temperature for heating soybeans, as indicated by the present investigation, is in agreement with the results reported by others. Parsons ('43), using rats, obtained the best product by autoclaving at 110°C. for 30 minutes, and Evans and McGinnis ('46), with chicks, obtained the best products by autoclaving 30 minutes at 100°C. or 110°C. Clandinin, Cravens, Elvehjem and Halpin ('46), however, reported that autoclaving for 4 minutes at 140°C. gave a good quality product. While differences in the heating techniques used experimentally would affect the products, it is believed that the evidence presented shows clearly that autoclaving at 108°C. is superior to 120°C., and that equally good results may be obtained at 108°C., whether the heating period is 15 or 30 minutes.

While the evidence presented supports the view that the trypsin inhibitor is the chief cause of the poor utilization of raw soybean protein, other data are required to explain the mechanism of its action. Since its action is manifested not only with soybean protein, but also with casein, and since Ham et al. ('45) showed that this inhibitor decreased the utilization of a practical ration by chicks, it appears that its action is not due to a specific configuration of the major proteins of the diet.

SUMMARY

1. A method was developed for determining the trypsin inhibitor potency of raw or heated soybean flours.

2. The protein efficiency of soybean flour increased in direct proportion to the destruction of its trypsin inhibitor potency by heat.

3. The protein efficiencies of autoclaved soybean flour to which the inhibitor had been added, and of isolated soybean protein partially freed of the inhibitor, agreed well with the values predicted from the results with heated flours. It was concluded that the presence of the trypsin inhibitor was the chief cause of the poor utilization of the protein or of inadequately heated soybean flours.

4. Autoclaving at 108°C. for 15 to 30 minutes produced a soybean flour of optimal protein efficiency for mice, while autoclaving at 120°C. for 5 or 10 minutes resulted in a slightly poorer product. Autoclaving for periods of over 10 minutes at 120°C. caused a progressive decrease in the protein efficiency of the flour.

5. On the basis of this evidence, it is suggested that the determination of the inhibitor potency of soybean products may be used as a practical index of the effectiveness of the heating to increase the protein quality, providing that the heating is not continued beyond the time required for the total destruction of the inhibitor.

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THE RELATION OF THIAMINE CLEARANCE TO BLOOD LEVELS IN MAN

M. C. BLANCHAER AND EILEEN CAMERON

The Nutrition Laboratory and Department of Medical Research, Medical School, University of Manitoba, Winnipeg, Canada

ONE FIGURE

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Since thiamine blood levels (Goodhart and Sinclair, '40) and urinary clearance values (Melnick and Field, '42) have been proposed as indications of thiamine status, it was thought desirable to study by both methods a number of subjects of varying nutritional sufficiency to determine the relationship between the results.

METHOD

Selection of subjects

The 23 subjects reported in this study were patients in the wards of a general hospital. Since it was impossible to obtain a reliable estimate of recent thiamine intake in each case, selection was directed solely toward securing a group with as wide a range of nutritional status as possible. Patients receiving sulfonamide drugs were excluded because of the possible adverse effect on the microbiological assay for thiamine used. The 2 estimations were repeated on 1 patient only. No cases of uncomplicated vitamin deficiency were available for study. Three of the subjects had been receiving thiamine therapy in the form of multivitamin tablets for varying periods before the estimations were made.

Test dose procedure and collection of specimens

After the subjects had emptied their bladders (8:30 A.M.), fasting specimens of blood were collected in heparin by venipuncture. Immediately after withdrawal of the blood, an intramuscular injection (gluteal) of 350 μg of thiamine chloride hydrochloride in saline per square meter of body surface was given.

Urine was collected covering a period of 4 hours after the injection, with the subjects in a fasting state. The brown collection bottles contained 10 ml of 5N HCl and a few drops of toluene. Since previous work in this laboratory had shown that a false low excretion may result from incomplete passage of a small specimen of very high thiamine concentration, water was given by mouth in an effort to obtain urine specimens of adequate volume.

Preparation of specimens and analysis

Blood specimens were centrifuged at 2500 R.P.M. for 20 minutes and the hematocrit values read. After the plasma had been pipetted off, the blood cells were resuspended in saline to the original volume of blood centrifuged. A 4-ml volume of the saline suspension of cells was pipetted into 80 ml of 0.5% acetate buffer, pH 4.5, containing 50 mg each of papain and takadiastase. This preparation was allowed to digest at room temperature for 24 hours and prepared for analysis as described by Sarett and Cheldelin ('44).

Upon completion of the collection period, the urine specimens were steamed to remove the toluene. An aliquot, depending on the volume and estimated content of thiamine, was brought to pH 6.5 and diluted to volume for analysis. Undiluted specimens were kept at —15°C. for additional analyses if necessary.

Both urine and blood specimens were analyzed for thiamine by the Lactobacillus fermentum method (Sarett and Cheldelin, '44; Cheldelin et al., '46). The pyrimidine and thiazole derivatives of the thiamine molecule had little if any activity under

the conditions of the collection and analytical method used. The sensitivity of the method enabled adequate dilution of urine specimens to prevent "drift" occasionally seen in this type of assay. Each specimen was set up for assay at 6 to 8 dilutions, together with recoveries of 0.005 μg added thiamine at 3 levels. All assays were checked for "validity" by the method of Wood ('45). A deviation of ± 10% from the mean result was considered the maximum permissible in any of the levels run, although usually the deviations fell within a ± 5% range.

The "P" value of Fisher ('32) was applied to correlation coefficients as a test of significance because of its suitability for small samples. A "P" value of 0.01 or less was required before a correlation was considered significant.

All references in this report to micrograms (μg) of thiamine should be read as "micrograms of thiamine chloride hydrochloride."

RESULTS

Physical measurements and laboratory findings are presented in order of thiamine excretion in table 1. Total thiamine excreted in 4 hours following administration of the test dose is plotted against blood cellular thiamine in figure 1.

Since Melnick and Field ('42) found clearance values below 50 μg invariably associated with thiamine deficiency, subjects excreting less than this amount were reviewed to test the possibility of dividing the series into 2 distinct groups. Although no clinical criteria for such a division could be arrived at, the thiamine clearance values of severely ill patients (subjects 16, 17, 18, 20, 21, 22 and 23) were generally low regardless of the etiology of their condition, while convalescent subjects and those in hospital for investigation of relatively mild conditions (subjects 1, 2, 5, 6, 7b and 9) usually excreted larger amounts of the test dose.

Rapidly changing food intakes made it difficult to assess the influence of this factor on the clearance results except in known cases of thiamine therapy. High levels of intake (10

to 20 mg thiamine per day) for a few days did not produce as high a clearance as might be expected (subjects 8 and 12), while an intake of 3 mg per day for a month was associated with an excretion in the "normal" range (subject 3).

TABLE 1

Data of present study.

SUBJECT NO.	AGE	WEIGHT	URINE VOLUME	THIAMINE EXCRETED	THIAMINE INJECTED	HEMATOCRIT	BLOOD CELLULAR THIAMINE	
							per 100 ml blood	per 100 ml cells
		lbs.	*ml*	*μg*	*μg*		*μg*	*μg*
1	35	134	330	139	580	35	3.8	10.8
2	34	138	285	130	600	49	12.8	26.0
3	65	142	160	115	580	37	12.3	33.2
4	53	160	190	111	610	45	9.0	20.0
5	77	160	995	111	640	48	6.8	14.2
6	18	170	390	87	690	49	7.0	14.2
7	34	112	850	75	540	41	5.3	13.0
7b	34	112	830	116	540	46	8.5	18.4
8	40	142	330	74	640	47	5.3	11.2
9	42	175	110	66	630	42	6.3	15.0
10	46	110	225	60	520	30	5.0	16.6
11	40	200	1230	57	680	45	7.5	16.6
12	13	96	70	53	490	50	8.8	17.6
13	75	150	720	48	580	54	6.5	12.0
14	66	143	85	44	570	45	7.1	15.8
15	60	128	135	37	570	46	8.5	18.4
16	74	106	170	34	530	45	7.3	16.2
17	79	92	870	30	480	34	4.5	13.2
18	71	98	180	25	520	46	10.0	21.8
19	37	97	283	23	500	42	9.5	22.6
20	55	131	65	20	600	35	7.3	20.8
21	50	107	145	13	750	42	9.8	23.4
22	75	122	55	9	540	41	11.3	27.6
23	64	152	220	6	630	25	4.5	18.0

The wide range of 10.8 to 27.6 μg thiamine per 100 ml of blood cells corresponded approximately to that reported for blood cocarboxylase in normal men by Goodhart and Sinclair ('39). High blood levels were found in subjects that excreted either large or small amounts of thiamine, with low levels

occurring mostly in those having intermediate clearance values of 60 to 100 μg. The highest and lowest blood levels were found in the group with clearance values in the "normal" range of 90 μg and over.

Consideration of the results suggested a division of the subjects into 2 groups (fig. 1): those who excreted less than 90 μg thiamine (group A) and those who excreted over this

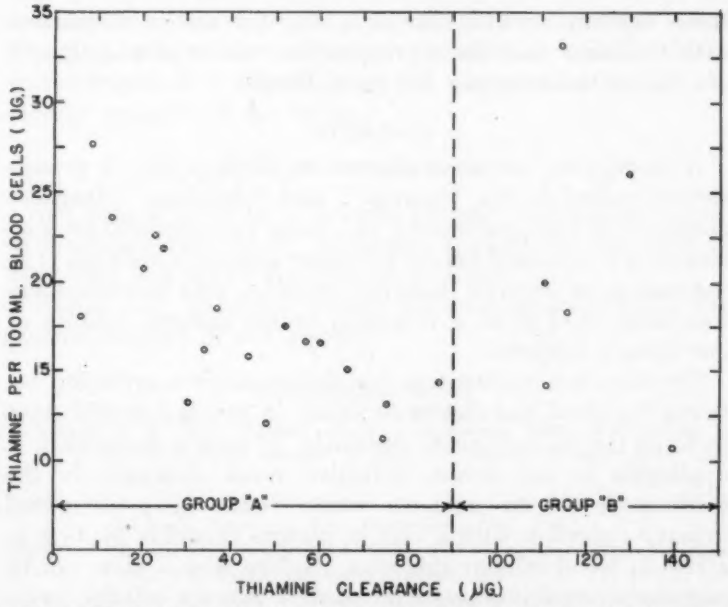

Fig. 1 Blood cellular thiamine of subjects plotted against thiamine clearance. Groups A and B are explained in the text.

amount (group B). While group B was too small for statistical analysis, it would seem that no relation existed between blood and excretion values. However, a statistically significant correlation coefficient of —0.74 was found for these values in group A. The corresponding correlation coefficient of —0.08 for groups A and B taken together was not significant. In addition, a significant correlation of —0.62

between age and thiamine clearance was obtained in group A, with only a moderately significant value (-0.37, $P = 0.05$) in groups A and B taken together. Further attempts to correlate thiamine output and blood levels with other variables such as weight and urine volume were unsuccessful.

It will be noted that blood levels are expressed throughout as micrograms of thiamine per 100 ml of blood cells. This is purely an arbitrary measure dependent upon the finding that these derived results showed a slightly better correlation with clearance than the corresponding values of micrograms of cellular thiamine per 100 ml of blood.

DISCUSSION

Although our series could not be divided into 2 groups corresponding to the "normal" and "deficient" clearance ranges of Melnick and Field ('42), there was a definite association of low clearance values with poor general condition. The correlation of lowered thiamine excretion with increased age was considered to be a reflection of the general debility of our elderly subjects.

The simplest explanation for the negative correlation between the blood and clearance values in group A would seem to be on the basis of renal clearance. If such a mechanism is applicable to our series, defective renal clearance in the debilitated, elderly subjects would result in a decreased urinary excretion with a rise in plasma thiamine leading to a rise in blood cellular thiamine. Before such a view can be accepted, it would be necessary to show that the cellular levels are dependent, at least in part, upon the plasma levels. This problem is being investigated.

Regardless of the physiological interpretation applied to these results, they do suggest that some caution should be exercised in using these methods for the classification of subjects into "normal" and "deficient" groups.

SUMMARY

1. Simultaneous blood thiamine and thiamine clearance estimations were made on 23 hospital patients.

2. Low clearance values were found in the more severely debilitated subjects regardless of the etiology of their condition, while larger amounts of thiamine were excreted by convalescent subjects and those under investigation for minor ailments.

3. In the group excreting less than 90 μg thiamine, higher blood levels were found in the more severely ill patients.

4. A significant negative correlation was found between blood cellular thiamine and thiamine clearance values below 90 μg. A similar correlation of age with clearance values was considered a reflection of the general debility of the elderly subjects of the series.

ACKNOWLEDGMENTS

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Merck and Company kindly donated cocarboxylase for standardization of the assay.

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